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Differential Gene Expression in a Louisiana Strain of Microalgae

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DIFFERENTIAL GENE EXPRESSION IN A LOUISIANA STRAIN OF MICROALGAE

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ABSTRACT

Considerable interest in alternative energy has stimulated research in biofuels, particularly microalgal biofuels. In particular, strains of algae that accumulate lipids to be used for biofuels must be adapted to outdoor growth and resistant to invasive species. Differential gene expression in a Louisiana algae/cyanobacteria co-culture consisting of approximately 97% *Chlorella vulgaris* and 3% *Leptolyngbya* sp. possessing these traits was examined. Possible reasons for the enhanced growth of the co-culture relative to a *Chlorella* monoculture were reviewed, including cyanobacterial symbiosis and chemicals produced by cyanobacteria or bacteria that could influence the growth of *Chlorella*.

The co-culture and *Chlorella* monoculture were cultivated at scalar irradiance levels of 180 and 400 μmol/m^2-sec and nitrate levels corresponding to 50% and 100% of the nitrate levels of Bold's Basal Medium. Dry biomass and cell counts were measured for the cultures initially, in the early exponential phase, in the late exponential phase, and at the end of the growth period. Lipid content was measured in the late exponential phase and at the end of the growth period. Total RNA was extracted and suppression subtractive hybridization was performed. Expressed sequence tags corresponding to putatively differentially expressed genes were sequenced, yielding one-hundred and five putatively differentially expressed genes. Quantitative PCR was performed on nine genes to compare gene expression in *Chlorella* in the co-culture and monoculture.

All nine genes showed statistically significant expression level differences between the *Chlorella vulgaris* in the co-culture and in the monoculture for cultures grown at the same irradiance and nitrate levels. Evidence from the gene expression experiments, combined with observations in the literature, suggest the possible effect of a cyanobacteria-produced substance

such as microcystins by the *Leptolyngbya* sp. Second, two Photosystem II genes were upregulated in the *Chlorella* monocultures, and one Photosystem I gene was upregulated in the co-cultures. Finally, the upregulation of a gene for an oil globule associated protein was found in the co-culture *Chlorella*. A homologous protein has been found in similar green algae, and further study of it in *Chlorella* is expected.

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Background

Since the discovery of petroleum, fossil fuels have grown to be an integral source of energy worldwide. More recently, considerable interest has been devoted to finding a new source of energy, both due to the scarcity of easily produced petroleum and geopolitical concerns regarding the location of many petroleum deposits (DOE 2010). Additionally, carbon dioxide levels in the atmosphere have sparked interest in ways to sequester the gas, which is released into the atmosphere through the burning of hydrocarbons and other sources.

One area of active research that has received considerable interest is that of fuels from microalgae. The Biomass Program of the U.S. Department of Energy (DOE) Office of Energy Efficiency spearheaded efforts to foster a sustainable biomass industry in the United States, combining the efforts of national laboratories, academia, and industry (DOE 2010). The Biomass Program discussed technological challenges to algal biofuels, including those of feedstock, conversion, and infrastructure. Although oil price dipped below $20 per barrel during 1995, near the end of the Aquatic Species Program, which took place from 1978 to 1996, (DOE 2010), rising oil prices, exceeding $140 per barrel during 2008, and climate change concerns have renewed interest in alternative sources of fuels. As of 2009, over 150 algal biofuels companies had been formed in the continued quest to make algal biofuels economical (DOE 2010). Furthermore, a study by the Department of Enerry's Pacific Northwest National Laboratory estimated that by choosing optimal location for production, up to 17% of the petroleum imported for transportation fuels could be replaced by biofuels (Wigmosta et al. 2011). The sustained efforts by government and industry to pursue biofuels are expected to continue in the future.

1.2 Advantages and Disadvantages of Algal Biofuels

Microalgal biofuels possess a number of advantages over other biofuels. First, microalgae have the ability to yield more energy than terrestrial crops per the same land area (DOE 2010), with theoretical yields to be estimated at 10-100 times per the same land area (Greenwell et al. 2009). Second, algae can be grown in environments not conducive to traditional terrestrial crop plants that are used for biodiesel production, such as soybeans, palm, sunflower, and rapeseed (Huang et al. 2010). Third, algae are able to be cultured using water sources such as wastewater, brackish water, and saline water, thus avoiding competition for precious water resources (DOE 2010). Furthermore, algae can even be grown in wastewater, removing nitrogen and phosphorus from the water (Tam and Wong 1990; Li et al. 2008).

Microalgal biofuels also have several desirable characteristics that conventional fuels such as diesel do not. For example, combustion of algal biofuels does not release as much nitrous oxide as does that of conventional fuels (Li et al. 2008), and algal biodiesel contains little to no sulfur (Vyas et al. 2010), resulting in less sulfur dioxide emissions as compared to combustion of conventional diesel (Demirbas and Demirbas 2010). Additionally, other products are produced by microalgae, further increasing the value of algal biomass. These bioproducts include pigments, such as the high-value carotenoid astaxanthin (Kang and Sim 2007), bioplastics (Zabochnicka-Swiatek 2010), omega-3 fatty acids such as eicosapentaneoic acid (EPA) and docosahexaneoic acid (DHA) (Harwood and Guschina 2009), and medicines and cosmetics (Zabochnicka-Swiatek 2010).

Nonetheless, algal biofuels currently remain far more expensive to produce than conventional fuels. While estimates of their cost vary widely, a study of several models for algal oil production cost estimation placed its cost at \$10.87 gal^{-1} to \$13.32 gal^{-1} (Sun et al. 2011). Three different models from 1982 to 1996, normalized to 2009 dollar amounts, estimated

biodiesel production costs at \$2.41 gal^{-1} to \$6.09 gal^{-1} (Gallagher 2011). The Algae 2020 study listed open pond biodiesel production costs of \$9 gal^{-1} to \$25 gal^{-1} and closed photobioreactor production costs of \$15 gal^{-1} to \$40 gal^{-1} (Thurmond 2009; Singh and Gu 2010). Nonetheless, cost models vary widely, based on factors such as cultivation setup, lipid yield of the algae, and the cost of inputs (water, nutrients, electricity, etc.) (Sun et al. 2011). Thus, intensive study has been devoted to every stage of the algal biofuels production process, including strain selection (Mutanda et al. 2011), algal cultivation (Demirbas and Demirbas 2010), enhancing lipid production (Courchesne et al. 2009), harvesting, and downstream production. The Department of Energy's *National Algal Biofuels Technology Roadmap* (DOE 2010) divides barriers to algal biofuels into three categories: feedstock, conversion, and infrastructure. Feedstock challenges are divided into the areas of algal biology (e.g., rapid screening of algal strains to determine those most suitable for biofuels production, improvement of strains by genetic engineering), algal cultivation (e.g., investigation and optimization of cultivation systems), and harvesting and dewatering. Conversion challenge categories are extraction and fractionation (e.g., comparison of processes and optimization of required process energy), fuel conversion, and co-products. Finally, infrastructure challenges include distribution and utilization and resources and siting (e.g., identification of ideal sites for algal production and maximal integration with producers of CO_2 and wastewater). All of these areas present research and development opportunities that will enhance the economic feasibility of algal biofuels.

1.3 Strain Selection and Algal Cultivation

The production of algal biofuels begins with the selection of a high-lipid producing strain and its cultivation. While extensive laboratory studies have demonstrated the ability of algal strains, including *Botryococcus braunii* and *Chlorella vulgaris* to have lipid contents of as high as 75 percent of the dry weight of the algae (Mutanda et al. 2011), these yields cannot

necessarily be achieved in large-scale cultures. Algae grown for lipid production can be cultivated photoautotrophically, in which CO_2 is the carbon source and light is required for growth; heterotropically, in which an organic carbon source is provided in the growth media and light is not provided; or mixotrophically, in which an organic carbon source, CO_2, and light are provided (Cheirsilp and Torpee 2012). Heterotrophic cultivation has the advantages of not requiring light and allowing easier control over the growth process (Prathima Devi et al. 2012) and can be performed in conventional bioreactors (Cheirsilp and Torpee 2012).

Table 1.1: Phototropic Algal Cultivation Systems: Advantages and Disadvantages (DOE 2010)

	Advantages	**Disadvantages**
Closed Photobioreactors	-Less loss of water than open ponds -Superior long-term culture maintenance -Higher surface to volume ratio can support higher volumetric cell densities	-Scalability problems -Require temperature maintenance as they do not have evaporative cooling -May require periodic cleaning due to biofilm formation -Need maximum light exposure
Open Ponds	-Evaporative cooling maintains temperature -Lower capital costs	-Subject to daily and seasonal changes in temperature and humidity -Inherently difficult to maintain monocultures -Need maximum light exposure

Phototropic growth of commercial mass algae cultures is usually done in either open ponds, which can be unmixed or mixed, with mixed ponds having a productivity of around 10 times higher (Ghasemi et al. 2012). Closed photobioreactors (PBRs) can also be used, with the key advantage that the culture is less prone to contamination (Sheehan et al. 1998; Rodolfi et al. 2009). A few types of PBRs, listed in Ghasemi et al. 2012, include stirred tank, bubble columns, and tubular PBRs. Some advantages and disadvantages each of open ponds and closed PBRs are

provided in Table 1.1 (DOE 2010). This table enumerates some of the key factors in algal cultivation, including appropriate temperature and light exposure, costs of the algal cultivation system, and the difficulty of maintaining monocultures.

Ideal algae cultivation combines strain selection with the correct cultivation system to address these aforementioned factors. One approach has been the use of native strains, which are adapted to outdoor growth in the environment in which they will be cultivated and would be more likely to withstand contamination by invasive species in outdoor ponds than would laboratory strains (Sheehan et al. 1998; Rodolfi et al. 2009). Thus, it has been recommended that optimization and genetic engineering studies begin with native strains (Sheehan et al. 1998; DOE 2010). By using genetically engineered native strains, the dual goals of preventing contamination of the culture while obtaining higher lipid yields could ideally be achieved (Rodolfi et al. 2009).

1.4 Engineering Algae for Lipid Production: Biochemical, Genetic, and Transcription Factor Engineering

Once an appropriate strain and algal cultivation system is selected, additional optimization for lipid production is done. Approaches to engineering algae for lipid production include biochemical engineering, genetic engineering, and more recently, transcription factor engineering (Courchesne et al. 2009).

Biochemical engineering approaches to maximize lipid production involve optimization of culture conditions, such as pH, temperature, irradiance, carbon source, aeration, and concentration of specific nutrients in the media (Courchesne et al. 2009). Nutrient starvation has been widely examined as a method of enhancing lipid production. Nonetheless, as nutrients are necessary for cell growth, a tradeoff exists between nutrient starvation producing a higher percentage of lipids per total biomass and the ability to produce more biomass, although with a

lower lipid percentage, without nutrient starvation (Courchesne et al. 2009). In particular, nitrogen is required for amino and nucleic acids as well as chlorophyll; phosphorus is required for ATP, DNA, and phospholipids; and iron is required for nitrogenase, cytochromes, and a wide variety of other enzymes (Graham and Wilcox 2000).

Studies focusing on *Chlorella* have investigated the effects of nitrate limitation (Illman et al. 2000; Converti et al. 2009; Bai et al. in review-b) and nitrogen limitation with urea as the nitrogen source (Hsieh and Wu 2009). Combining nitrogen depletion with an optimal CO_2 concentration and harvesting the algae at different times can also optimize growth and lipid productivity (Widjaja et al. 2009). The effects of nitrate, phosphorous, and combined nitrate and phosphorous limitation on *Chlorella vulgaris* have also been measured (Multu et al. 2011). The effects of adding iron once or twice with or without the addition of nitrate were compared, and the addition of iron was found to prolong the exponential growth phase (Liu et al. 2008). Temperature also influences lipid productivity, and one study found comparable growth but with a higher lipid content when *Chlorella vulgaris* was grown at 25°C rather than 30°C (Converti et al. 2009). Finally, the influence of irradiance level on photosynthesis rate (Bhola et al. 2011) and growth and lipid productivity (Bai et al. in review-b) has been examined.

Considering the large number of past studies on nutrient starvation as far back even as the 1950s or 1960s (DOE 2010), this technique provides a relatively easy way to enhance lipid concentrations in microalgae. Additionally, the use of lower concentrations of nutrients lowers the cost of culturing the algae, an additional advantage, especially in the quest to make the process economical for large-scale lipid production. Nonetheless, while nutrient starvation can increase lipid concentration, it hinders growth; thus, the balance between growth to accumulate biomass, combined by high lipid content in the biomass, is important to overall lipid

productivity.

The second set of approaches to lipid overproduction use genetic engineering. Recent progress has been made in the identification of fatty acid biosynthesis genes and in microalgae (Khozin-Goldberg and Cohen 2011; Merchant et al. 2011; Rismani-Yazdi et al. 2011). In particular, the synthesis pathways to the production and accumulation of triacylglycerols (TAG), has been constructed (Merchant et al. 2011). Triacylglycerols are molecules possessing three acyl chains of 16 to 22 carbon atoms each, and TAGs are converted into fatty acid methyl ester (FAME) in the production of biodiesel (Demirbas and Demirbas 2010). Following pathway determination, researchers have focused on either overexpressing enzymes involved in TAG biosynthesis (Courchesne et al. 2009) or blocking biochemical routes that channel TAG precursors into other product. One particular area of focus has been inhibition of starch synthesis, with the goal of channeling fixed carbon into lipids instead (Wang et al. 2009; Li et al. 2010). Interaction between starch and lipid synthesis in microalgae is expected to be investigated further (DOE 2010).

Key to genetic engineering studies of microalgae are sequenced genomes and gene transfer mechanisms. Eukaryotic algae with sequenced genomes include *Chlamydomonas reinhardtii*, *Dunaliella salina*, and *Ostreococcus taurii*. One of the most important eukaryotic algal strains sequenced is *Chlorella variabilis* NC64A, the sequencing of which was completed in 2010 (Blanc et al. 2010). Gene transfer mechanisms have been developed for a number of common algae species, including *Chlamydomonas*, *Dunaliella*, and *Chlorella* (Dawson et al. 1997), as well as cyanobacteria species, including *Synechocystis*, *Synechococcus*, *Anabaena*, and *Nostoc* (DOE 2010; Radakovits et al. 2010). Knowledge of algal genomes as well as gene transfer mechanisms will foster genetic engineering and yield insight into possible lipid

overproduction methods, but care must be taken in preventing the release of genetically modified strains into the environment (DOE 2010).

Transcription engineering approaches are the third and most nascent set of methods for stimulating lipid accumulation in microalgae. Rather than merely overproducing or blocking the production an enzyme, transcription factor engineering (TFE) would provide a way to regulate multiple enzymes (Courchesne et al. 2009). Transcription factors identified in *Chlorella variabilis* NC64A are listed in the Plant Transcription Factor Database (http://plntfdb.bio.uni-potsdam.de/v3.0/) (Perez-Rodriguez et al. 2009). Nonetheless, work in this area is nascent, and widespread use of TFE for industrial lipid production in microalgae will probably not be viable for some time.

1.5 The Louisiana Strain Co-Culture

The present study focuses on a native strain of algae isolated from near Louisiana State University. Drs. Rusch and Gutierrez-Wing at Louisiana State University collected a sample from College Lake, located southeast of the university at 30.406703 N, 91.170135 W. An environmental sample was collected from the lake, from which a hardy co-culture consisting of *Chlorella vulgaris* and *Leptolyngbya* sp. was obtained (Rusch and Gutierrez-Wing in review). TEM and SEM images of the co-culture are presented in Figure 1.1 (Bai et al. in review-a).

Subsequent attempts to separate the microalgae and cyanobacteria into monocultures yielded lackluster growth, but the co-culture of these two species, consisting of approximately 3% *Leptolyngbya* sp. by cell count, displayed excellent growth. Preliminary studies of the *Chlorella vulgaris* in the co-culture showed cell counts of approximately 20 times those of the *Chlorella vulgaris* monoculture. Additionally, the co-culture resisted contamination while the monoculture was eventually contaminated by fungi. The reason why both the *Leptolyngbya* sp. and *Chlorella vulgaris* grew better in the co-culture is unknown.

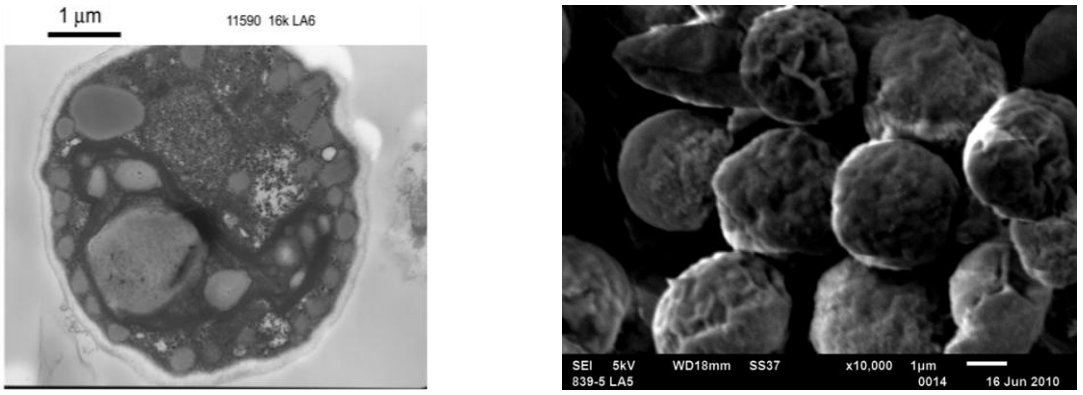


Figure 1.1: TEM (left) and SEM (right) images of the Louisiana *Chlorella vulgaris*/*Leptolyngbya* sp. co-culture. Note the presence of lipid globules in the TEM image.

The proliferation of cells in the co-culture was accompanied by production of a viscous exudate that was not present in the cyanobacteria or microalgal monocultures (our unpublished data). The exudate makes the process of extracting lipids from the co-culture biomass significantly more difficult, clogging the filter used for this process. Both the composition and source of the exudate are unknown, although the exudate is speculated to contain polysaccharides.

1.6 Differential Gene Expression Analysis: Suppression Subtractive Hybridization

Because functions in the cell are controlled by gene expression, measuring genes provides vital information regarding the status of the cell. Differential gene expression analysis refers to the measurement of genes that are expressed differentially between cells at two different conditions. By measuring gene expression, an understanding of cell processes, and how they are regulated under different cell growth conditions, can be gained.

A variety of techniques have been developed to analyze differential gene expression; several of them are discussed in Farrell 2006. One more commonly used technique is suppression subtractive hybridization. Suppression subtractive hybridization (SSH) is a

technique used to identify upregulated or downregulated genes sequences by comparing two pools of DNA and to exponentially amplify the differentially expressed sequences (Diatchenko et al. 1996; Farrell 2009). The technique is able to identify genes that are up- or downregulated by as little as 50% between two pools of DNA in some cases (Farrell 2009). One key advantage of SSH is that the technique does not require the genome of the species being studied to be sequenced, and thus it can be used for novel gene discovery (Diatchenko et al. 1996). SSH has been applied to study gene expression in several strains of microalgae, including *Dunaliella salina* (Zhang et al. 2002), *Synechococcus* (Jones et al. 2006), and *Chlorella vulgaris* (Machida et al. 2008).

1.7 Objective

The Louisiana strain co-culture possesses the desirable properties of resistance to invasive species and adaptation to the light, humidity, and temperature conditions of the local environment. Nonetheless, why the co-culture possesses superior growth and productivity, displaying larger cells and increased biomass levels, compared to that of the *Chlorella vulgaris* monoculture is unknown. The objective of the current study is to determine the gene expression differences between *Chlorella vulgaris* in the Louisiana strain co-culture and the *Chlorella vulgaris* in the monoculture.

One reason the enhanced growth and productivity of the co-culture may include the formation of a *Chlorella vulgaris*/*Leptolyngbya* sp. symbiosis. Another might be the production of growth-promoting substances in the culture media benefitting one or both species. Such substances, including plant growth substances (PGS), polyamines, and polysaccharides, could be synthesized by either of the two species and excreted into the media.

In this study, suppression subtractive hybridization will be used to examine differential gene expression between the *Chlorella vulgaris* in the co-culture and in the monoculture. Genes

that are upregulated and downregulated in the *Chlorella vulgaris* in the co-culture compared to that in the monoculture will be identified for culture conditions corresponding to a 2x2 factorial experiment, with two treatment levels of irradiance and two treatment levels of starting nitrate concentration in the media. Differentially expressed genes will be sequenced, and their sequences will be compared to genes in database, including genes of green algae and higher plants. Finally, levels of expression of selected genes in the different culture conditions will be examined using quantitative PCR (qPCR).

CHAPTER 2: REVIEW OF LITERATURE

2.1 Introduction

The present work focuses on differential gene expression in the Louisiana strain *Chlorella vulgaris*/*Leptolyngbya* sp. co-culture compared to the *Chlorella vulgaris* monoculture. Since the co-culture has been studied for lipid productivity, the pathways and genes involved in lipid will be examined, with a focus on microalgae. Second, since a symbiosis between the two species is possibly involved in the co-culture, cyanobacterial symbioses will be reviewed. Although cyanobacteria form symbioses with a wide variety of organisms, those between cyanobacteria and microalgae or higher plants are the most pertinent to this work. Third, since *Leptolyngbya* sp. or bacteria in the co-culture might produce a chemical substance that benefits the *Chlorella vulgaris*, plant growth substances known to be produced by bacteria or cyanobacteria and known to have an effect on *Chlorella* (both *Chlorella vulgaris* and other species in the genus *Chlorella*) will be discussed. Finally, mixed cultures including algae and cyanobacteria and/or bacteria will be reviewed.

2.2 Lipid Production Pathways and Genes

Important to the ability of microalgae to produce and accumulate triacylglycerols (TAG) is an understanding of the pathways and genes involved. A diagram showing the metabolic pathways involved in biofuel and biofuel precursor production is provided in Figure 2.1 (Radakovits et al. 2010). Nonetheless, this work focuses on the microalgae grown to produce lipids, so only the fatty acid synthesis pathways will be discussed in detail.

Fatty acid biosynthesis begins with acetyl-CoA. Conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase is an essential step because it commits the fixed carbon into a pathway resulting in fatty acids (Courchesne et al. 2009). A basic diagram of the fatty acid and triacylglycerol biosynthesis pathway is provided in Figure 2.2 (Courchesne et al. 2009).

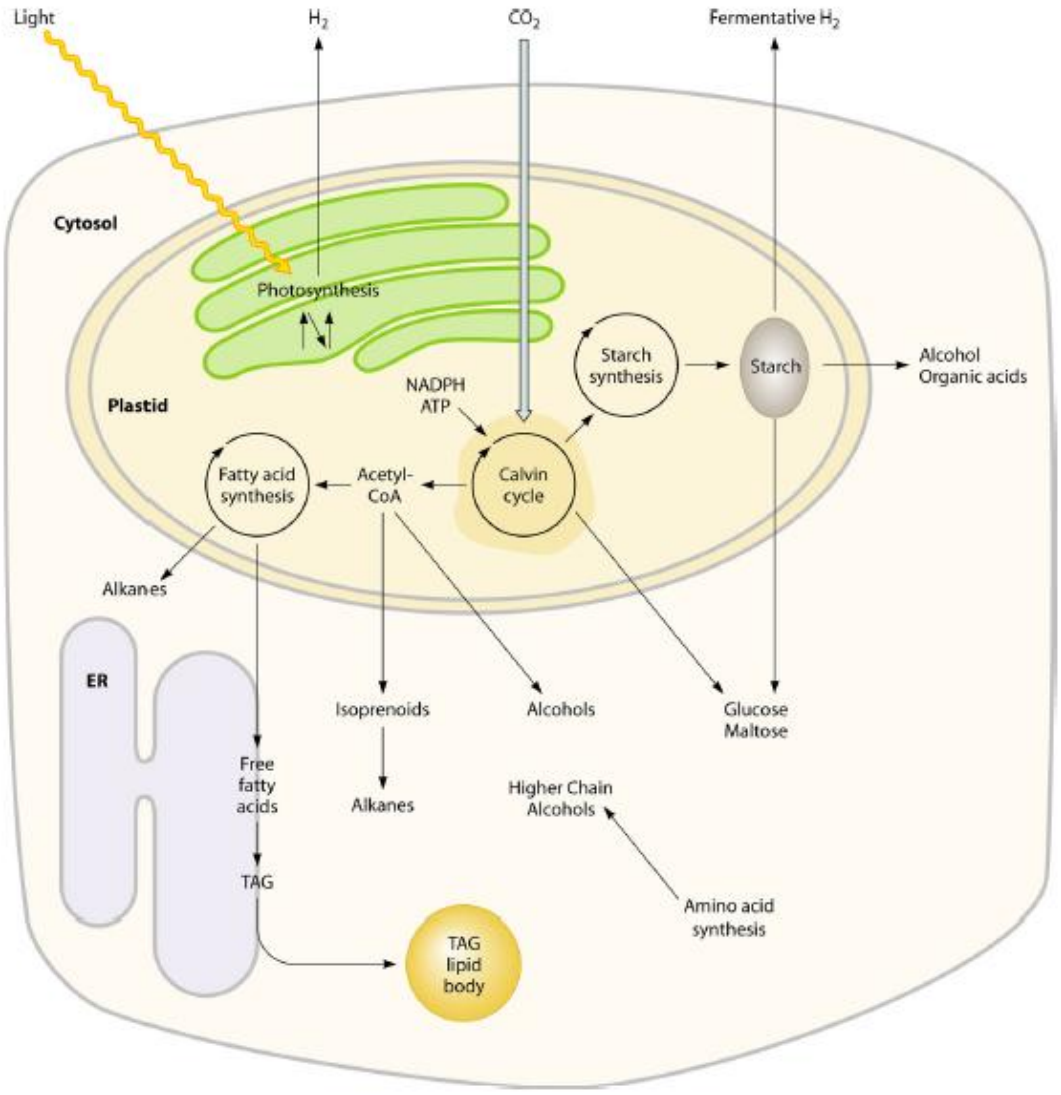


Figure 2.1: Metabolic pathways in algae that can be used to produce biofuels or biofuel precursors (Radakovits et al. 2010)

Detailed pathways for algal fatty acid biosynthesis are provided in Rismani-Yazdi et al. 2011, including the pathway for starch synthesis, which competes for fixed carbon with FA synthesis. Pathways specific to polyunsaturated fatty acids are provided in Harwood and Guschina 2009. Knowledge of the pathways also provides information regarding the types of fatty acids an organism is capable of producing, an important factor in the selection of algae for

biofuels to meet American (ASTM D6751) and European (EN 14214) standards for biodiesel (Knothe 2011).

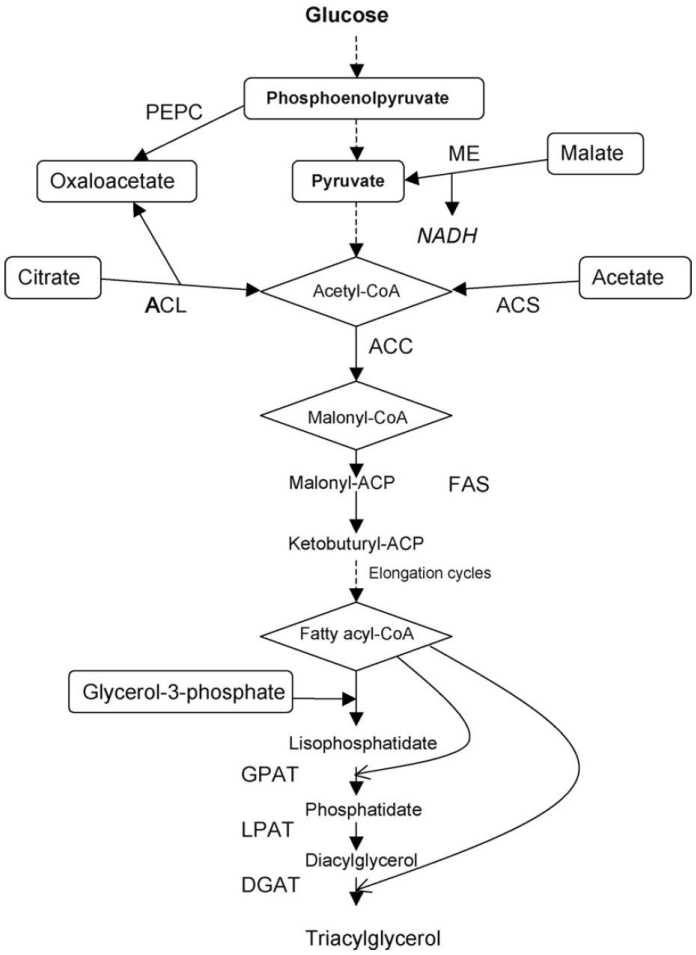


Figure 2.2: Pathways for fatty acid and triacylglycerol biosynthesis (Courchesne et al. 2009). Abbreviations for enzymes are as follows: PEPC, phosphoenolpyruvate carboxylase; ME, malic enzyme; ACL, ATP:citrate lyase; ACS, acetyl-CoA synthase; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthetase; GPAT, glycerol-3-phosphate; LPAT, lysophosphatidate acyl-transferase; DGAT, Acyl-CoA:diacylglycerol acyl-transferase.

Much recent work has been done in identification of fatty acid (FA) biosynthesis genes in green algae. A discussion of FA genes in eukaryotic algae is provided in Khozin-Goldberg and

Cohen 2011. Species-specific reviews of FA synthesis genes have been performed on *Chlamydomonas reinhardtii* (Merchant et al. 2011) and *Dunaliella tertiolecta* (Rismani-Yazdi et al. 2011). Although the genome of *Dunaliella tertiolecta* has not been sequenced, the sequencing of its transcriptome was sufficient to identify homologous genes to those known for sequenced organisms, including *Volvox carteri*, *Chlamydomonas reinhardtii*, and higher plants (Rismani-Yazdi et al. 2011). Using a similar approach, fatty acid biosynthesis pathways in other green algae could be enumerated.

2.3 Cyanobacterial Symbioses

Cyanobacteria are known to enter different types of symbiotic arrangements with a wide variety of other organisms from all of the taxonomic kingdoms, including bacteria, microalgae, fungi, higher plants, and invertebrates. Nonetheless, only particular genera of cyanobacteria are known to form symbioses, which require the cyanobacterium to adapt to its host (Rasmussen and Nilsson 2002). A symbiosis is a biological association of two or more organisms in which each organism derives some benefit from, is harmed by, or appears to have no effect from the association (Paracer and Ahmadjian 2000). A discussion of different cyanobacterial symbioses will lend insight into their symbiotic relationships in general and in particular the possible symbiosis between *Chlorella vulgaris* and *Leptolyngbya* sp. in the co-culture.

2.3.1 Cyanobacterial Symbioses—Algae

Relationships between bacteria and algae either involve an association between bacteria and microalgae, bacteria and macroalgae, or algae present in benthic microbial mats (Graham and Wilcox 2000). Of the first type, most cyanobacteria associations with microalgae are with diatoms (Janson 2002). Both cyanobacteria and microalgae excrete organic substances, including amino acids, carbohydrates, and lipopolysaccharides, which provide carbon and nitrogen to heterotrophic bacteria in proximity (Graham and Wilcox 2000). In the association,

the bacteria could provide a variety of benefits to the algae, including lowering oxygen concentrations to benefit nitrogen fixation, nitrogen fixation, providing growth factors, or producing Fe, CO_2, NH_4^+, NO_3, or PO_4^{3-} (Graham and Wilcox 2000). Thus the microalgae and bacteria in close proximity provide a variety of mutual benefits to each other.

The second type of bacteria-algae relationship, bacteria with macroalgae, does not necessarily involve cyanobacteria, but nitrogen-fixing cyanobacteria have been observed to grow on the green macroalga *Codium* (Carpenter and Foster 2002). When bacteria are involved, they are believed to provide a similar function as in the first type, providing substances used by macroalgae (Graham and Wilcox 2000). The third classification, benthic microbial mats, involve autotrophic cyanobacteria and diatoms providing O_2 and dissolved carbon sources for heterotrophic bacteria (Graham and Wilcox 2000). One study documents the formation of spheres within these microbial mats, which involved *Phormidium* cyanobacteria, the diatom *Navicula perminota*, and heterotrophic bacteria (Brehm et al. 2003). Filaments formed by the cyanobacteria trapped the diatoms inside, which multiply until the sphere is filled and then leave; the space inside the sphere and the sphere's surface both have extracellular polymeric substances present (Brehm et al. 2003).

Cyanobacteria are known to associate with diatoms, both intracellularly and extracellularly (Janson 2002). In the intracellular symbioses, the cyanobacteria most likely provides fixed nitrogen for the diatom (Janson 2002). Likewise, some of the extracellular associations involve heterocysts, or cyanobacteria cells created specifically to fix nitrogen; in some cases, the cyanobacteria may also fix carbon for the diatom (Janson 2002). A third type of association involves cyanobacteria, the protozoan *Solenicola setigera*, and the diatom *Leptocylindrus mediterraneus*; *Synechocystis* cells are attached to a matrix formed by the other

two symbionts (Janson 2002). In this particular association, empty diatom frustules suggest that the protozoan feeds on the diatom (Carpenter and Foster 2002).

While the present study does not involve diatoms, the most common microalgal symbiont of cyanobacteria, several characteristics of these associations are of interest. First, cyanobacteria are known to provide nitrogen fixation and perhaps even carbon fixation in symbiosis and may receive dissolved organic substances from the symbiont as well. Second, extracellular cyanobacterial-algal symbioses often involve a structure that promotes close proximity for the association, such as the filamentous structure of the microbial spheres or the matrix to which the *Synechocystis* cells attached in the tripartite symbiosis with the diatom, cyanobacteria, and protozoan. Extracellular polymeric substances present may play a role in the attachment and/or provide a nutrient source for a heterotrophic species in the association.

2.3.2 Cyanobacterial Symbioses—Lichens

Perhaps the best known cyanobacterial symbioses are the lichens, of which there are approximately 17,000 species, mostly terrestrial but with at least 7 marine species known (Graham and Wilcox 2000; Carpenter and Foster 2002). The cyanobacterium provides fixed carbon and sometimes fixed nitrogen to the fungus, with the symbiosis varying from mutualistic to the fungus being parasitic (Graham and Wilcox 2000). Only 14-15% of lichens contain cyanobacteria; the far majority involve green algae instead (Graham and Wilcox 2000). Of the cyanobacterial genera involved in symbioses, the widest variety are involved in lichens (Graham and Wilcox 2000). Nonetheless, their study is not as important as that of the symbioses between cyanobacterial and terrestrial plants.

2.3.3 Cyanobacterial Symbioses—Terrestrial Plants

A variety of terrestrial plants form symbioses with nitrogen-fixing cyanobacteria (Graham and Wilcox 2000). The symbiosis usually involves the plant providing carbon to the

cyanobacteria in exchange for receiving fixed nitrogen (Graham and Wilcox 2000). The vast majority of known cyanobacterial-plant symbioses involve the *Nostoc* species (Adams 2002).

Two necessary factors for cyanobacteria-plant symbioses are heterocysts and homogonia (Adams 2002). Heterocysts, or cyanobacterial cells specifically developed to fix nitrogen, supply nitrogen to both the other cyanobacterial cells and the plant, while homogonia are motile structures that provide the blue-green algae access to the area of the plant at which the symbiosis takes place (Adams 2002). Establishment of the symbiosis requires the alga to attach to the plant, a process that may be aided by extracellular polysaccharides (Adams 2002). The plant often excretes a substance to enhance growth of the homogonia, such as the hormogonia-inducing factor (HIF) that is synthesized by nitrogen-starved hornworts; other plants are known to produce analogous substances for this purpose (Adams 2002).

Evidence of the cyanobacteria providing fixed nitrogen to hornworts and liverworts includes an increased frequency of heterocysts in the symbiosis as opposed to free-living conditions (Adams 2002). Nonetheless, the cyanobacteria still assimilates sufficient nitrogen, and its nitrogen reserves in the forms of phycobiliproteins and cyanophycin remain intact, whereas they would be metabolized if the cyanobacteria needed nitrogen (Adams 2002). Likewise, the blue-green algae receive carbon from the plant, most likely as sucrose, which is sometimes stored as glycogen, the storage suggesting that they contain ample carbon resources (Adams 2002).

Cycads, a family of seed plants including the sago palm (*Cycas revoluta*), are also known to harbor symbiotic cyanobacteria in their roots. (Costa and Lindblad 2002). Filamentous cyanobacteria involved in the symbioses are found in mucilage (Costa and Lindblad 2002). The cyanobacteria are involved in nitrogen fixation, as determined by a $^{15}N_2$ experiment that verified

the transfer of fixed nitrogen from the *Nostoc* to the cycad in the form of citrulline or glutamine (Costa and Lindblad 2002). Lacking exposure to light in the root system, the cyanosymbionts obtains carbon either from the host plant or via dark cycle CO_2 fixation (Costa and Lindblad 2002).

Cyanobacteria in the genus *Nostoc* form a symbiosis with the plant *Gunnera*, the only angiosperm currently known to form a symbiosis with a nitrogen-fixing cyanobacteria (Bergman 2002). Unlike in other terrestrial plant symbioses, the *Gunnera* hosts the cyanosymbionts intracellularly (Bergman 2002). *Gunnera* species are not naturally found without the *Nostoc* cyanosymbiont, of which multiple strains have been found even within a single plant (Bergman 2002). To stimulate the infection process, *Gunnera* emits hormogonia inducing factor, as in the aforementioned hornworts that have symbiotic cyanobacteria (Bergman 2002). A mucilage, containing carbohydrates and arabinogalactan proteins (AGPs), is secreted by the plant and is believed to play a role in the symbiosis, and uninfected glands, particularly those near freshly growing regions of the plant, release the most mucilage (Bergman 2002). The cyanobacteria is heterotrophic, obtaining not only carbon but also other nutrients from the plant, particularly Fe for the synthesis of the enzyme nitrogenase (Bergman 2002).

Another symbiosis between higher plants and cyanobacteria involves the nitrogen-fixing *Anabaena* with the water fern *Azolla*, allowing it to grow without an additional nitrogen source (Graham and Wilcox 2000). *Azolla* can be grown without the cyanobacterial symbiont through the addition of chemical fertilizer (Wagner 1997), but the primary cyanosymbiont is not known to grow outside of the symbiosis or with other symbiotic plant species (Gusev et al. 2002).

A final cyanobacterial symbiosis category involving terrestrial plants is the attempted induction of artificial symbioses, both to aid in nitrogen-fixation and to study interactions

between plants and cyanobacteria (Gusev et al. 2002). One concern is the ability of the introduced cyanobacterial species to compete with extant microorganisms; a strategy to overcome this problem is to introduce a mixed culture rather than a single strain, with the added benefit that mixed cultures synthesize more growth hormones and enzymes than monocultures (Gusev et al. 2002). Another factor influencing symbiosis is the necessity of proximity between symbionts, which is either provided by the cyanobacteria being located in plant compartment or guaranteed by attachment through an extracellular mucous (Gusev et al. 2002). In the case that the two species' cells are not in direct contact, the plant provides exocellular agents that are responsible for hormogonia formation, nitrogen assimilation regulation, and changes in the cyanobacteria (Gusev et al. 2002). Substances produced by the cyanobacteria for the plant include fixed nitrogen products and plant growth substances (Gorelova 2006). Thus, the cyanobacteria-plant association involves a complex interchange of chemical substances, even when direct contact between symbiont cells is not present.

An examination of the wide variety of symbioses into which cyanobacteria enter reveals many common traits. First, in most cases the cyanosymbiont provides nitrogen fixation for the other partner, and the use of a $^{15}N_2$ isotope tracer is one established method to test nitrogen fixation, as in (Costa and Lindblad 2002). Second, in the cases in which the cyanosymbiont is located extracellularly, a mucilage is often associated with the symbiosis. Third, particularly in higher plants, chemical signaling takes place between the host and cyanobacteria in establishing the symbiosis. The chemical signals are able to diffuse through media in situations in which the cells of the two symbionts are not in direct contact. All three of these characteristics are important to the study of the *Chlorella vulgaris*/*Leptolyngbya* sp. co-culture. One final point of

interest is the increased synthesis of growth substances and enzymes in mixed cultures as compared to monocultures (Gusev et al. 2002).

2.4 Plant Growth Substances in Microorganisms

One possible reason for the enhanced growth of the Louisiana strain co-culture could be the presence of plant growth substances (PGS) in the exudate. While the possibility that plant hormones also control algal growth has been considered for several decades, the mid-1980s marked a resurgence in the field (Tarakhovskaya et al. 2007). In addition, the development of more accurate analytical techniques such as gas chromatography (GC), mass spectroscopy (MS), high-performance liquid chromatography (HPLC), and nuclear magnetic resonance spectroscopy (NMR) have provided more accurate methods of identification than bioassays (Bradley 1991; Evans and Trewavas 1991). With renewed interest in the growth of algae for biofuels and the continuing quest to use microorganisms to produce the growth substances for crop plants, several reviews of plant growth substance production in microorganisms have been published recently (Tsavkelova et al. 2006; Tarakhovskaya et al. 2007). Nonetheless, challenges remain, particularly in studying organisms in association, in which determining the organism that synthesized the substance is crucial (Evans and Trewavas 1991).

A plant hormone is a plant-produced organic substance that regulates processes such as growth, development, and differentiation at concentrations lower than those of nutrients (Bradley 1991; Davies 2010). For higher plants, the following set of seven criteria are used to ascertain if a hormone is the cause of a process or response (Bradley 1991):

1. The chemical is located and measured at the location of response.
2. Removal of the hormone production site stops the effect.
3. Response can be restored by subsequent reapplication of the hormone.
4. If the cell or cells can be isolated, *in vitro* response and *in vivo* response should be the same.
5. Presence of the substance in similar cases.
6. The chemical produces a specific response to be able to observe a causality

relationship.
7. The effect will be different in an organism that has a mutation in the hormone-controlled process.

While the structural differences between plants and microalgae make use of some of the criteria difficult, the list provides insight into the design of experiments to study the response of phytohormones in algae. In the case of a co-culture, the fourth criterion proves difficult because the plant hormone response may only be present in the co-culture and not in species monocultures.

Table 2.1: The Ten Phytohormone Groups
(adapted from Tarakhovskaya et al. 2007)

Name	Biosynethtic Pathway	Basic Physiological Activity
Auxin	From tryptophan or indole	Induction of elongation growth; differentiation of phloem elements; apical dominance; trophisms; initiation of root formation; etc.
Cytokinins	Biochemical modification of adenine	Control of cell division; bud development; development of the leaf blade; senescence retardation
Gibberellins	From glyceraldehydes-3-phosphate	Stem elongation; initiation of seed germination
Ethylene	from methionine	Senescence induction; initiation of defensive responses
Abscisic acid (ABA)	From carotenoids	Control of the stomatal apparatus function; growth inhibition; seed dormancy
Polyamines	By decarboxylation of arginine or ornithine	Regulation of growth and development at micromolar concentrations
Brassinosteroids	From mevalonic acid	Control of division, growth by elongation, differentiation of the vascular system
Jasmonides (oxilipins)	From polyunsaturated fatty acids	Development of defensive responses
Salicylates	From phenylalanine	Induction of the complex of defensive responses during pathogenesis

While researchers disagree on the groups of plant hormones, several groups of chemicals are shared among different lists. At least the following five substances are considered plant hormones: auxins, gibberellins, cytokinins, ethylene, and abscisic acid (ABA) (Tsavkelova et al. 2006). The other possible groups are polyamines, brassinosteoids, jasmonides (oxilipins),

salicylates, and signal peptides (Tarakhovskaya et al. 2007). The ten phytohormone groups, their biosynthetic pathways, and their basic physiological activity in higher plants are listed in Table 2.1 (adapted from Tarakhovskaya et al. 2007).

Although algae lack many of the structures and functions present in higher plants in which the plant hormones are involved, algae and other microorganisms have nonetheless been found to contain plant hormones. A variety of heterotrophic prokaryotes, phototrophic prokaryotes, eukaryotic algae, and fungi have been found to contain auxins, gibberellins, or cytokinins (Tsavkelova et al. 2006).

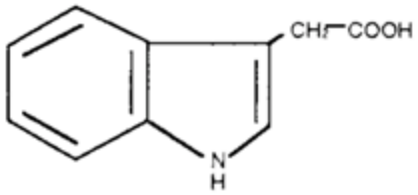
2.4.1 Auxins

Auxins are plant hormones that play an important role in plant growth. The chemical structure of the most common auxin, indole-3-acetic acid (IAA), is shown in Figure 2.3. The amino acid tryptophan has an identical structure, except for the insertion of a $CH\text{-}NH_2$ between the CH_2 and COOH groups of IAA, and most auxins share the indole structure also present in tryptophan (Tsavkelova et al. 2006). At least five auxin production pathways have been identified in rhizobacteria, bacteria that live in association with plant roots (Glick et al. 1999). Some organisms are known to synthesize auxins using multiple pathways (Costacurta and Vanderleyden 1995).

The study of auxins in algae is generally done either through comparison to the auxin signaling system in higher plants or through the addition of auxins to algae growth media. In the latter case, variables such as dry weight and chlorophyll content in the algae grown in the present in the media containing auxins are compared to values of the same for a control sample grown in media without the auxins.

An investigation of ten strains of algae (including *Chlorella* sp. NC64A and six other

green algae) did not reveal any of the three components of the auxin signaling pathway found in the genomes of all investigated terrestrial plants (Lau et al. 2009). These include the auxin-indole-3-acetic acid proteins (AUX-IAA), transport inhibitor response1-auxin signaling F-box protein (TIR1-AFP), and auxin response factor (ARF) (Lau et al. 2009). Nonetheless, a gene orthlogous to auxin-binding protein 1 (ABP1) was identified in *Chlorella variabilis* NC64A (Blanc et al. 2010).



Indole-3-acetic acid

Figure 2.3: Chemical structure of the most common auxin, IAA (Glick et al. 1999)

Two *Chlorella* studies examined the effect of auxins, auxin precursors, and auxin analogues on several variables (Czerpak et al. 1994; Piotrowska et al. 2008). Seven different auxins or auxin precursors increased the dry weight and chlorophyll, carotenoids, and protein contents of *Chlorella pyrenoidosa* grown in media containing the substances (Czerpak et al. 1994). Additionally, indomethacin (IM), a substance containing an indole ring that has a chemical structure similar to IAA, has been found to promote *Chlorella vulgaris* growth and metabolism (Piotrowska et al. 2008).

Of particular interest is whether cyanobacteria have the ability to produce IAA or another

auxin. A study of axenic cultures of cyanobacteria, using ELISA (enzyme-linked immunosorbent assay) performed with an anti-IAA antibody, confirmed that some strains of cyanobacteria do synthesize IAA (Sergeeva et al. 2002). More definitive analytical results using ELISA and GC-MS verified the production of IAA. Further testing used a DNA probe for the gene *ipdC*, which codes for indolepyrovate carboxylase, and a positive test in 4 of the 11 strains tested suggested that IAA could be synthesized using the indole-3-pyruvic acid pathway (Sergeeva et al. 2002). The use of ELISA and GC-MS for analysis, as done in this study, is preferred over non-specific bioassay methods because it allows for more accurate results and specific identification of compounds.

Furthermore, the demonstrated use of DNA probes for presence of the *ipdC* gene suggests that similar tests could be conducted to test for any of the enzymes involved in IAA biosynthesis, provided that sufficient DNA sequence information is known for that particular enzyme. Identification of a gene related to auxin synthesis and verification that the enzyme for which it codes is being expressed would provide important evidence that auxins are being synthesized.

The studies done on algae suggest promising results in the ability of IAA and its precursors and analogues to stimulate growth and metabolism. Nonetheless, much work remains to be done in the area, including determination of auxin production pathways in various strains and studies measuring whether auxins have an effect on the cytoplasmic lipid production of microalgae.

2.4.2 Cytokinins

Cytokinins are compounds derived from adenine that influence cell division (Roberts and Hooley 1988). The chemical structures of two common cytokinins, kinetin and zeatin, are

shown in Figure 2.4. Note that they share the same basic structure with the exception of the substitution at the N6 site.

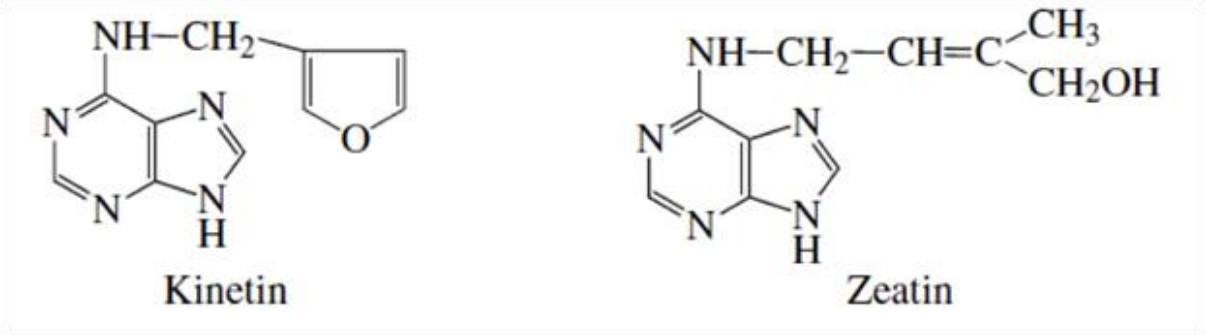


Figure 2.4: Chemical structures of the cytokinins kinetin and zeatin (Tsavkelova et al. 2006)

In higher plants, cytokinins regulate a variety of processes, including adaptation to stress and activation of RNA and protein synthesis (Tsavkelova et al. 2006). A variety of hererotrophic bacteria, phototrophic prokaryotes, and eukayotic algae synthesize cytokinins (Cacciari et al. 1989; Costacurta and Vanderleyden 1995; Trotsenko et al. 2001; Tsavkelova et al. 2005; Tsavkelova et al. 2006).

Cytokinins are believed to be produced from tRNA degradation in algae (Tarakhovskaya et al. 2007), but in various bacteria biosynthesis occurs from isopentyl pyrophosphate and 5'-AMP using the enzyme isopentenyltransferase (Tsavkelova et al. 2006). A gene coding for isopentenyltransferase has been identified in the plant *Arabidopsis thaliana*, but other *de novo* pathways exist in plants (Tsavkelova et al. 2006). Several other cytokinins are listed in Tarakhovskaya et al. 2007 and Stirk et al. 2003. Cytokinins are classified into isoprenoid and aromatic types, based on the type of substituted molecule at the N^6 site, and both have been found in algae (Stirk et al. 2003; Tarakhovskaya et al. 2007).

Two experiment studies involving cytokinins have been performed with *Chlorella*, and cytokinin synthesis genes orthologous to those of model plant *Arabidopsis* were found in the

genome *Chlorella variabilis* NC64A (Blanc et al. 2010). The addition of cytokinins to the growth media increased levels of monosaccharides, chlorophyll, and carotenoid production in *Chlorella vulgaris*, suggesting an effect of cytokinins on photosynthesis (Pietrowska and Czerpak 2009). Three different auxins and three different cytokinins, when added to the growth media of *Chlorella pyrenoidosa*, were found to increase the content of protein and chlorophyll relative to a control (Czerpak et al. 1999). The effects of the auxins were more pronounced than the effect of the cytokinins.

As with the auxin studies performed on microalgae, an important aspect of future studies will be whether cytokinins influence the production of lipids. Since lipid productivity is driven by amount of biomass produced by a particular algae culture, the role of cytokinins to promote cell growth is of particular interest.

2.4.3 Other Plant Growth Substances

Genes believed to be involved in the synthesis of abscisic acid (ABA), polyamines, brassinosteroids were also found in *Chlorella variabilis* NC64A (Blanc et al. 2010). Abscisic acid (ABA), a plant hormone whose structure is shown in Figure 2.5, is known to stimulate plant growth in low concentrations. In higher plants, it is generally considered to be involved in stress response, initiation of dormancy, and closure of the stomata (Mauseth 1998). ABA is produced in all photosynthetic organisms (Cutler and Korchko 1999), but in *Chlorella* its purpose is not well understood (Tarakhovskaya et al. 2007). Nonetheless, one study demonstrated increased oxygen for dark respiration as well as increased nitrogen uptake with ABA in *Chlorella fusca* (Ulrich and JKunz 1984).

The effects of the diamines agmatine and putrescene and the polyamines spermine and spermidine have been measured in *Chlorella vulgaris* (Czerpak et al. 2003a). At least 3 of the 4 substances increased at least one of the following: chlorophyll content, monosaccharides content,

and cell count. Additionally, a variety of cyanobacterial strains are known to synthesize polyamines (Hosoya et al. 2005). Since polyamines could certainly be dissolved in the exudate produced in the *Chlorella vulgaris*/*Leptolyngbya* sp. co-culture, the ability of diamines and polyamines to enhance *Chlorella* growth and metabolism is important.

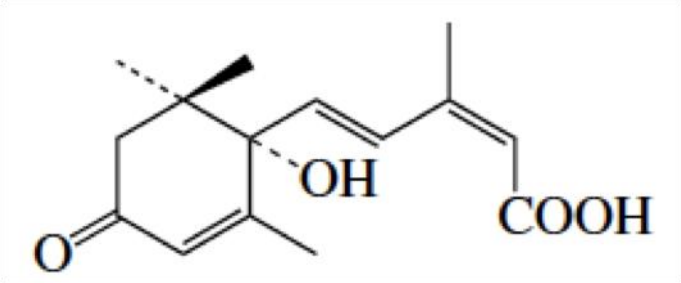


Figure 2.5: Chemical structure of abscisic acid (Czerpak et al. 2003b)

Brassinosteroids (BRs) are a group of phytohormones first discovered in 1976 that are believed to control RNA and protein synthesis as well as stimulate stem growth and mediate stresses in higher plants; they have the ability to increase the proton concentrations in the growth medium (Bajguz and Czerpak 1996; Bajguz 2000).

The structure of the first brassinosteroid discovered, brassinolide, is shown in Figure 2.6. The effects of brassinosteroids and their interaction with auxins and other phytohormones in higher plants has been studied in-depth, but very little work has been done in microalgae (Bajguz and Czerpak 1996). Brassinosteroids were found to increase cell count and protein content when added to the growth media of *Chlorella vulgaris* (Bajguz 2000). Additionally, *Chlorella vulgaris* is known to produce at least 7 brassinosteroids (Bajguz 2009).

Another phytohormone found in *Chlorella* is jasmonic acid, the structure of which is shown in Figure 2.7 (Tarakhovskaya et al. 2007). Jasmonic acid (JA) has a variety of functions in higher plants, including signaling for stress response and accumulation of free fatty acids

(Czerpak et al. 2006). JA was also identified in other green microalgae as well as the cyanobacterium *Spirulina* (Ueda et al. 1991a; Ueda et al. 1991b), and jasmonic acid methyl ester was found in *Chlorella* (Czerpak et al. 2006).

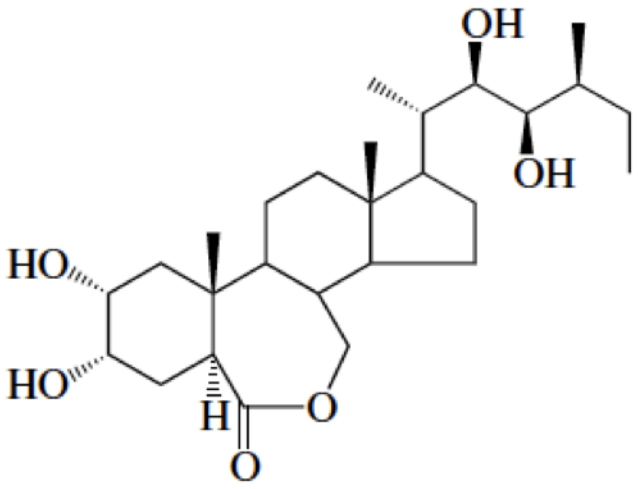


Figure 2.6: Chemical structure of brassinolide (Bajguz and Czerpak 1996)

Addition of jasmonic acid to the growth media of *Chlorella vulgaris* was found to increase cell number, carotenoids and chlorophyll content, and the amount of excreted protein, and it is believed to be involved in stress response (Czerpak et al. 2006).

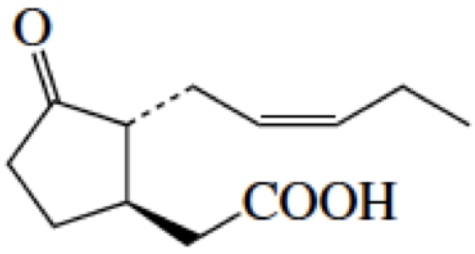


Figure 2.7: Chemical structure of jasmonic acid (Tarakhovskaya et al. 2007)

Jasmonic acid and its methyl ester are interesting as possible components of the co-culture exudate because JA is known to be produced by cyanobacteria and promotes fatty acid accumulation in higher plants.

2.4.4 Synopsis of Plant Hormones

An examination of several groups of plant hormones reveals that while many have positive effects on the growth of algae, with some studies being performed on *Chlorella* strains, their possible involvement in the *Chlorella vulgaris*/*Leptolyngbya* sp. co-culture would require that at least one of the species or the bacteria in the culture synthesize and excrete them. Analytical techniques such as LC-MS could be used to test for the possible presence of these substances in the growth media.

2.4.5 Polysaccharides

Additional substances that are most likely present in the co-culture exudate are monosaccharides or polysaccharides. Both cyanobacteria and microalgae are known to produce polysaccharides that are present on the outside of the cell (exopolysaccharides, or EPS) or secreted into the cell growth medium (released polysaccharides, or RPS). Over one-hundred strains of exopolysaccharide-releasing cyanobacteria have been studied, with the exopolysaccharide in the forms of shealth, capsules, or slimes (De Philippis et al. 2001). Renewed study in the last decade has focused not only on the RPS but also on the remaining biomass, which could be used to bind heavy metals in aqueous environments (De Philippis et al. 2001). Proposed uses of the EPSs include food additives, bioflocculants, and thickening agents; in addition, they increase the water-retaining ability of soil (De Philippis et al. 2001; Yu et al. 2010). Finally, sulfated polysaccharides possess antiviral and antitumor properties that could be exploited for pharmaceuticals (Otero and Vincenzini 2003; Yu et al. 2010).

External polysaccharides are believed to form a protective barrier between the cell and

the environment, both for microalgae (Mohamed 2008) and cyanobacteria (De Philippis et al. 2001; Yu et al. 2010). One study exposed *Chlorella vulgaris* and *Scenedesmus quadricula* to microcystins, toxic substances produced by cyanobacteria. While the growth and cell density of the algae was hindered for the first three days of microcystin exposure, within fourteen days the exposed algae had recovered to a cell density approximating that of the control cultures (Mohamed 2008). The algae produced polysaccharides that were able to trap reactive oxygen species produced due to microcystin-induced stress; the antioxidant capability of the polysaccharides of *Scenedesmus* exceeded that of those of *Chlorella* due to the higher sulfated content of the former (Mohamed 2008). Additionally, RPS from the algae were present in the culture medium, and an increased toxin concentration was correlated with increased RPS concentration (Mohamed 2008). While the RPS are proposed to be responsible for detoxification or reduced uptake of the microcystins, they can bind heavy metal ions and may stimulate the release of substances useful to the algae from heterotrophic bacteria that use the RPS as a carbon source (Mohamed 2008).

Because the co-culture exudate may contain polysaccharides and both the *Leptolyngbya* sp. and the *Chlorella vulgaris* are capable or synthesizing and excreting them, it may be difficult to determine which species produced the exudate, particularly if it is not present in monocultures of either species. Nonetheless, isotope tracer studies may provide a means to determine which species produced the exudate.

2.5 Mixed Cultures

A number of mixed culture studies have been performed using *Chlorella* sp. Several of them focus on the co-culturing of *Chlorella* with nitrogen-fixing bacteria. Co-immobilization of *Chlorella vulgaris* with the nitrogen-fixing bacteria *Azospirillum brasilense* increased growth of the *Chlorella* compared to that of a *Chlorella* monoculture (Gonzalez and Bashan 2000).

However, co-immobilization with the nitrogen-fixing bacteria *Phyllobacterium myrsinacearum* yielded no increase in cell count or biomass for the *Chlorella* (Gonzalez-Bashan et al. 2000). Finally, co-immobilization with nitrogen-fixing strain *Bacilus pumilus* promoted the growth of *Chlorella vulgaris* (Hernandez et al. 2009).

Many studies have focused on the use of mixed cultures to remove nutrients from wastewater. A co-culture of *Chlorella vulgaris* and cyanbacterium *Planktothrix isothrix* was able to remove more ammonia from a treated wastewater effluent than monocultures of either species (Silva-Benavides and Torzillo 2011). Industrial wastes were treated using a mixed culture of *Chlorella vulgaris* and oleaginous yeast *Rhodotorula glutinis*; a possible reason for the enhanced growth was the production of oxygen by the *Chlorella* and its use by the yeast, in addition to the production of CO_2 by the yeast and its use by *Chlorella* (Cheirsilp et al. 2011).

Finally, interest has been devoted to strains of bacteria that grow in *Chlorella* cultures. Co-cultured bacteria can influence *Chlorella* growth both positively and negatively, depending on the strain (Vu et al. 2010). Eight bacterial strains isolated from a culture of *Chlorella ellipsoidea* all appeared to enhance *Chlorella* growth; the strain that most promoted the growth was determined to be of the genum *Brevundimonas* (Park et al. 2008). Another study characterized the variety of bacteria found in a non-axenic culture of *Chlorella vulgaris*; one of the strains found in the culture was of the genus *Brevundimonas* as well (Lakaniemi et al. 2011). Advancement in technologies that can be used to quickly identify bacteria in non-axenic cultures, such as quantitative PCR as used in Lakaniemi et al. 2011, combined with an interest of promoting growth of microalgae, will be likely to drive additional studies of mixed cultures of microalgae, whether to produce lipids, to remove nutrients from wastewater, or for other purposes.

2.6 Conclusion

The present work focuses on differential gene expression in the Louisiana strain *Chlorella vulgaris*/*Leptolyngbya* sp. co-culture compared to the *Chlorella vulgaris* monoculture. With the goal of better understanding the lipid productivity of the culture, lipid biosynthesis pathways have been reviewed. Second, cyanobacterial symbioses, of which there are a wide variety, have been examined, with the goal of identifying common traits among them. Third, plant growth substances, which might be produced in the cultures, have been described. Finally, a number of studies involving mixed cultures of *Chlorella* sp. with nitrogen-fixing bacteria, other bacteria, and yeast have been enumerated. These four themes combine to shed light on the Louisiana strain co-culture on which this work is focused.

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

For this study, the Louisiana strain co-culture and a monoculture was cultivated. A flow chart summarizing the materials and methods is provided in Figure 3.1. The materials and methods will be discussed below, with more detailed descriptions provided in Appendix A.

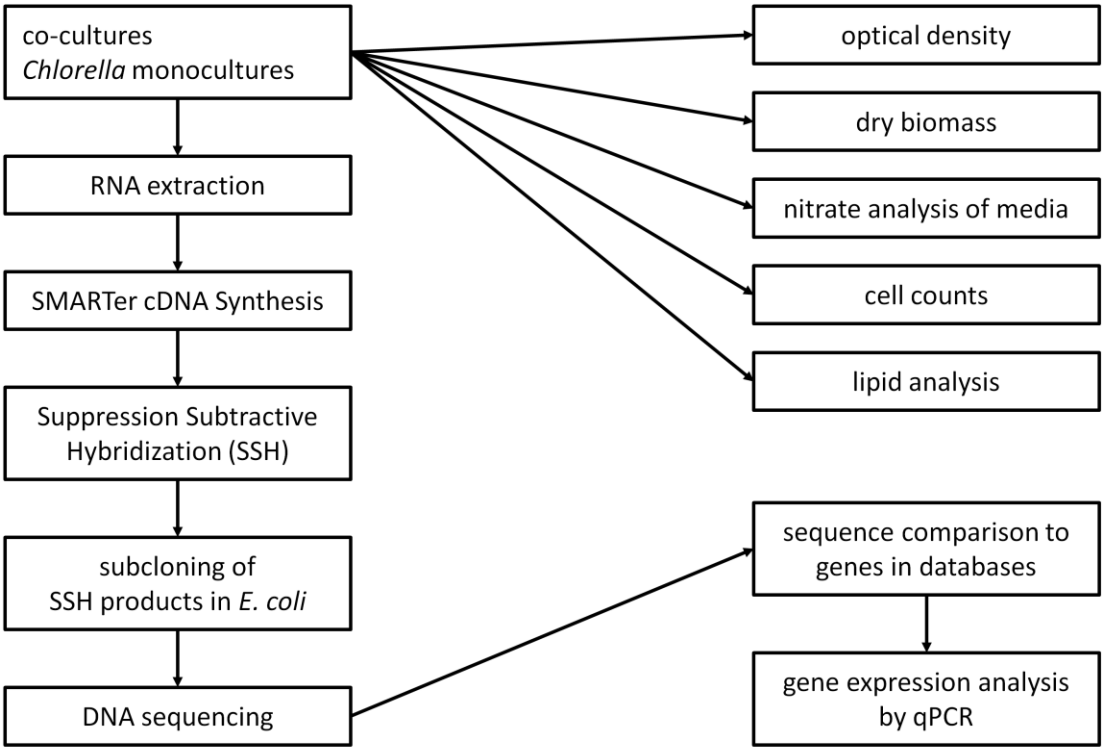


Figure 3.1: Overview of the materials and methods for the entire project.

Unless otherwise specified, all water used in the experimental procedures was deionized and filtered to attain a resistivity of 18.2 MΩ-cm, sterilized by UV radiation, and 0.2 µm filtered using either a Direct-Q 3 (EMD Millipore, Billerica, Massachusetts) or Nanopure Infinity (Barnstead Nanopure, Dubuque, Iowa) water system. All reagents were molecular biology or ACS grade. For the experiments involving RNA, aerosol filter piper tips and disposable

plasticware was used whenever possibly prevent the introduction of RNases, enzymes which degrade RNA, to the samples. Additionally, the laboratory bench and surfaces of equipment used was wiped down with RNase AWAY™ (Invitrogen, Grand Island, New York) to control RNases.

3.2 Algae Strains

The Louisiana strain co-culture was isolated from an environmental sample collected from College Lake, located near Louisiana State University at 30.406703 N, 91.170135 W. Isolation of the co-culture from the environmental sample was performed by Drs. Rusch and Gutierrez-Wing (Rusch and Gutierrez-Wing in review). The isolated strain was determined to consist of a *Chlorella vulgaris* and *Leptolyngbya* sp., as determined by Dr. David Nobles at UTEX The Culture Collection of Algae at the University of Texas. Sequencing of the internal transcribed spacer region 2 (ITS2) between the 18S and 5.8S rDNA was used for identification of the *Chlorella*, and sequencing of the 23S rDNA was used for identification of the *Leptolyngbya*. The percentage of *Leptolyngbya* cells in the co-culture ranges from approximately 3-7%, with a higher percentage of cyanobacteria when the culture was grown under lower levels of irradiance.

To perform a comparative genetic study between the *Chlorella vulgaris* in the co-culture and the monoculture, the *Chlorella vulgaris* was isolated. Successive dilutions of a co-culture sample were plated onto nutrient agar (BD Difco, Franklin Lakes, New Jersey) with a w/v composition of 1.5% agar, 0.3% beef extract, and 0.5% peptone. Single-species colonies were viewed under a light microscope, and those judged to be unialgal for *Chlorella vulgaris* were selected using a pipet connected to a vacuum. Note that the term *Chlorella vulgaris* monoculture will be used to describe a culture in which *Chlorella vulgaris* is the only algae present, although the culture is not axenic. In fact, algal cultures have been observed to have better growth with

some bacterial contamination, possibly due to the bacteria providing vitamins or other growth factors (Andersen 2005). Approximately 70 colonies were collected to guarantee appropriate genetic variation in the isolated monoculture. Agar plate cultures were then transferred to liquid media. Liquid cultures of the *Chlorella vulgaris* monoculture and the co-culture were grown in Bold's Basal Medium with Vitamins (BBM+V), prepared according to the Sammlung von Algenkulturen Göttingen recipe (Göttingen 2008). Starter cultures were grown under fluorescent lighting and continually aerated using ambient air passed through an 0.3 µm air filter (Aquatic Eco-Systems, Apopka, Florida) and an ultraviolet light sterilizer.

For the remainder of this work, the co-culture will be abbreviated CC, and the *Chlorella vulgaris* monoculture will be abbreviated Chl. For the purpose of clarity, the system of notation presented in Table 3.1 will be used in this work.

Table 3.1: Nomenclature for Algae Cultures

Culture	Scalar Irradiance Level (µmol/m^2-sec)	Starting Nitrate Level in the Media (% of nitrate level in Bold's Basal Medium)	Nomenclature
Chl	180	50	180Chl50N
Chl	180	100	180Chl100N
Chl	400	50	400Chl50N
Chl	400	100	400Chl100N
CC	180	50	180CC50N
CC	180	100	180CC100N
CC	400	50	400CC50N
CC	400	100	400CC100N

3.2 Growth Conditions

3.2.1 General Environment

The CC and Chl cultures were cultivated indoors in plastic carboys. The three-gallon (11.36 L) carboys, readily available at retailers in the United States and purchased locally, originally contained drinking water. The carboys were of a light blue color, transparent, and

composed of polycarbonate plastic. Prior to use for algae culture, the carboys were washed using a laboratory detergent, sterilized by chlorination, and rinsed.

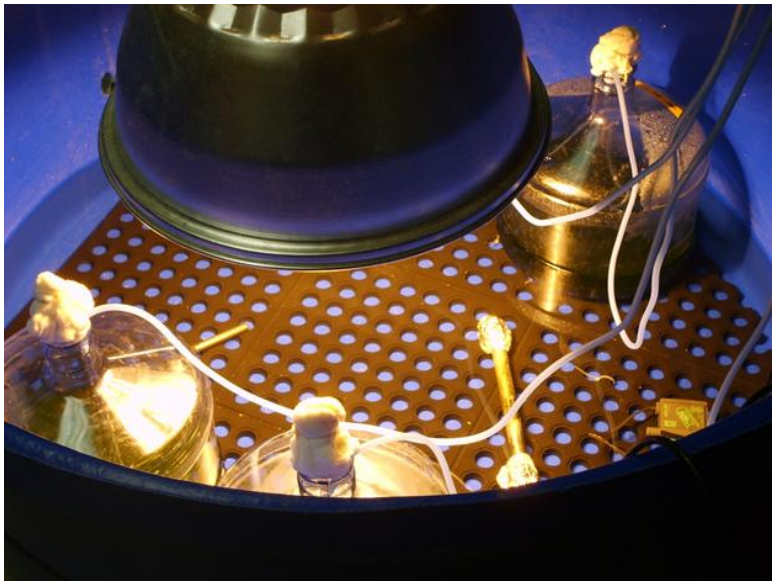


Figure 3.2: Culture environment for the 5-L carboy cultures, showing the blue polyethylene tub, a circulating temperature-controlled water bath, aerated cultures in 5-L carboys, and the lamp.

A total of eight cultures were grown, four of the CC and four of the Chl. The algae cultures were grown in a circulating water bath in a blue polyethylene tub. The culture environment is shown in Figures 3.2 and 3.3. Controlled variables included the algae media composition (BBM+V, with 50% and 100% of the specified nitrate levels for the medium), incident irradiance (87 and 204 µmol/m^2-sec, corresponding to 180 and 400 µmol/m^2-sec scalar irradiances), aeration rate (45 L/h), carbon dioxide aeration rate (9.4 L/day), pH (maintained between 6.5 and 8.0), and temperature of the circulating water bath (29±1°C). The cultures were grown according to a 2x2 factorial design, with two different levels of incident irradiance and two different levels of nitrate concentration in the media.

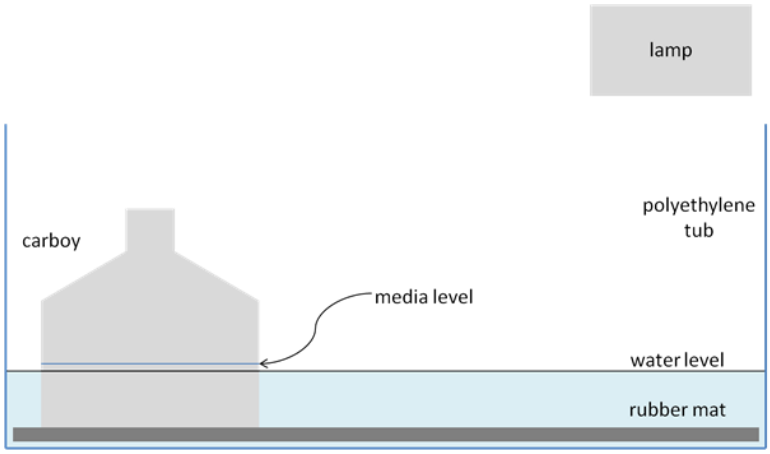


Figure 3.3: Diagram of culturing environment for the 5-L carboy cultures.

The four CC were grown at the same time. The four Chl cultures were also grown simultaneously, but at a different time than the CC cultures were grown. Growing each set of cultures at the same time was done in an effort to reduce variability.

3.2.2 Culture Media

Growth medium was prepared according to the SAG recipe (Göttingen 2008) with only the modifications listed below. A stock solution with five times the concentration of the six salt solutions specified in the recipe was prepared and autoclaved. Afterward, water was added to dilute the media. The trace metals solution was prepared separately and autoclaved, and the vitamins were supplied from stock solutions that were filter sterilized.

Separate stock solutions were prepared for the media that had 50% and 100% the nitrate levels of Bold's Basal Medium. For the 50% nitrate media, one-half the specified amount of the $NaNO_3$ stock solution and the same volume of water were added instead of the volume of the $NaNO_3$ stock solution specified in the recipe.

Algae cultures were inoculated to have an optical density value, measured as an absorbance at 664 nm, of less than 0.200. The volume of the culture inoculum was less than

one-fifth the total volume of the 5-L of the carboy cultures, and the Bold's Basal Medium was prepared as if the inoculum culture had no residual nutrients. To obtain homogenous inoculum samples for each of the four cultures, the flask was shaken before measuring out the inoculum volume for each carboy culture.

3.2.3 Lighting

The cultures received continuous irradiance from a high-pressure sodium (HPS) lamp mounted above the culture. The amount of light reaching the algae was reduced by the distance from the lamp, light absorbed by the carboy and the media, and light absorbed within the algae culture. Irradiance measurements in $\mu mol/m^2$-sec of photosynthetically active radiation (PAR) were taken using a LI-1400 Datalogger (LI-COR Biosciences, Lincoln, Nebraska) using a LI-190 flat quantum sensor and a LI-193 spherical quantum sensor. Cultures were grown at a low-light condition of 87 $\mu mol/m^2$-sec and a high-light condition of 204 $\mu mol/m^2$-sec, as measured by the LI-190 flat quantum sensor. Corresponding scalar irradiances, as measured by the LI-193 sensor, were 180 $\mu mol/m^2$-sec and 400 $\mu mol/m^2$-sec, respectively. The cultures will hereafter be referred to by their scalar irradiances of 180 $\mu mol/m^2$-sec and 400 $\mu mol/m^2$-sec. The scalar irradiance measurements were taken in the carboys, with the sensor immersed in 5 L of water.

Additional measurements were taken with the LI-190 flat quantum sensor. Since the flat sensor was not immersible in liquid, irradiance measurements for water, Bold's Basal Medium, and algae cultures were taken with the sensor inside the carboy at the level of the culture and below the bottom of the carboy with the fluid inside. The drop in irradiance due to the bottom of the carboy was taken into account in computing how much light penetrated the algae culture. The following incident irradiance measurements were taken each for the high-light and low-light culture conditions:

1. On top of the rubber mat in the polyethylene tub, without the carboy present

2. Inside the carboy at the liquid level of the cultures (the incident irradiance of the culture)
3. Without the carboy at the liquid level of the cultures
4. Below an empty carboy
5. Below a carboy containing 5L of water
6. Below a carboy containing 5L of media
7. Below the carboy containing three different dilutions of algae culture

The first six of the above light measurements for the high-light and low-light conditions are summarized in Table 3.2; measurements for the seventh condition are presented in Tables 3.3 and 3.4.

Table 3.2: Incident Irradiance Measurements (Flat Quantum Sensor) for the High-Light and Low-Light Culture Conditions

Measurement Number	Irradiance for High-Light Condition (µmol/m^2-sec)	Irradiance for Low-Light Condition (µmol/m^2-sec)
1	309	162
2	204	87
3	325	135
4	143	67
5	131	61
6	131	61

Note that the water and the media caused the same amount of reduction in irradiance at both light conditions. Three additional light measurements were taken for three different dilutions each of the CC and Chl cultures. Five-liter cultures of each were grown to approximately equal optical density values, taken as an absorbance at 664 nm. The irradiance was measured below the carboy for the cultures at one-times, two-times, and four-times dilutions to ascertain how much light penetrates the algae cultures at different culture densities of algae. The measurements, including the optical density values of the dilutions of algae for 664 nm and 750 nm, are presented in Tables 3.3 and 3.4. Absorbance due to chlorophyll was measured at 664 nm (Rodrigues et al. 2011), and turbidity was measured using the 750 nm absorbance (Fischer et al.

2010). Also provided is how much the irradiance drops in the algae cultures in the carboys compared to carboys containing media without algae.

Table 3.3: Irradiance Measurements for CC Cultures of Three Different Dilutions

Algae Dilution and Light Condition	OD (664 nm)	OD (750 nm)	Irradiance Measurement (µmol/m^2-sec) Below the Carboy	Irradiance Reduction (µmol/m^2-sec) Due to the Algae Culture
1, high	0.869	0.748	2.17	129
2, high	0.434	0.410	12.5	119
4, high	0.227	0.216	26.6	104
1, low	0.869	0.748	0.83	60.2
2, low	0.434	0.410	5.23	55.8
4, low	0.227	0.216	11.4	49.6

Table 3.4: Irradiance Measurements for Chl Cultures of Three Different Dilutions

Algae Dilution and Light Condition	OD (664 nm)	OD (750 nm)	Irradiance Measurement (µmol/m^2-sec) Below the Carboy	Irradiance Reduction (µmol/m^2-sec) Due to the Algae Culture
1, high	0.876	0.714	1.74	129
2, high	0.494	0.425	10.7	120
4, high	0.254	0.227	32.2	98.8
1, low	0.876	0.714	0.71	60.3
2, low	0.494	0.425	4.77	56.2
4, low	0.254	0.227	13.8	47.2

Even at an irradiance of approximately 0.250, most of the irradiance reduction observed in more dense cultures is already observed.

The average irradiance at different depths in a culture of green algae for HPS lamps was determined to follow the exponential function. At a fixed depth, the decrease in average irradiance due to increasing levels of biomass in the culture was also found to follow an exponential decay curve (Benson 2003; Benson and Rusch 2006).

3.2.4 Aeration and pH Control

Ambient air was supplied to the cultures through sterile glass 5 ml pipets. The ambient air was fed by an aquarium pump through vinyl or silicone aquarium tubing that was sterilized

by chlorination, and the air feed was 0.3 µm-filtered to prevent introduction of airborne organisms into the cultures. The carboys were sealed with a plug consisting of cotton gauze wrapped around cotton balls and tied with cotton string; the plugs were sterilized before use by autoclaving. The use of cotton plugs allowed for gas exchange while preventing airborne microorganisms from falling into the cultures.

The algae cultures were continuously aerated at a flow rate of 1.6 SCFH, or approximately 45 L/h. Carbon dioxide was fed for 1 minute at a flow rate of 28 L/h (1 SCFH, 9.4 L/day) through the same feed as the air every 72 minutes to control the pH of the cultures. Feeding CO_2 served to maintain the pH of the cultures in the ideal range of 6.5 to 8.0, and an additional dose of CO_2 was provided if the pH was measured to be above 8.0. The pH of each culture was measured twice daily using an Orion 420A pH meter (Thermo Scientific, Pittsburgh, Pennsylvania) and recorded.

3.2.5 Temperature Control

Culture temperatures were controlled by a circulating water bath. Tap water was poured into the polyethylene tub, as shown in Figure 3.2. A 300-W aquarium heater connected to a temperature controller (Aqua Logic, San Diego, California) heated the water bath when necessary to maintain a water temperature of 29±1ºC. This temperature was attainable without chilling the water bath due to the air conditioning in the building in which the algae was cultured. A water pump, oriented to direct water flow perpendicular to the long axis of the cylindrically shaped aquarium heater, circulated the water. The temperature of the water bath was measured twice per day and recorded. Tap water was added to the water bath daily to maintain a water bath level near that of the algae culture level, and the water in the bath was chlorinated daily by the addition of 5 ml of 6% NaOCl solution to reduce the growth of microorganisms that could contaminate the cultures.

3.3 Algal Culture Growth Data Collection

3.3.1 Optical Density

Optical density measurements were collected for each culture twice daily. The culture was vortexed to break up any clumped cells and homogenize the culture in the case of settling, and 1000 µl of the culture was pipetted into a disposable poly(methyl methacrylate) spectrophotometer cuvette. The absorbance of the algae culture was measured at 664 nm and 750 nm in a Beckman Coulter DU730 Life Science UV/Vis Spectrophotometer (Brea, California). The device was blanked using Bold's Basal Medium. When the 664 nm optical density value exceeded a value of 1.000, absorbance values of two-times and four-times dilutions of the culture were prepared as well.

3.3.2 Dry Biomass Determination

Dry biomass measurements of the culture were taken according to Environmental Engineering/Water Quality Laboratory Standard Operating Procedure (EiEL/Water Quality Laboratory SOP) PA 200, Total Suspended Solids Freshwater Algae Matrix, which is based on Standard Methods for the Examination of Waste and Wasterwater 2540D (APHA 1999).

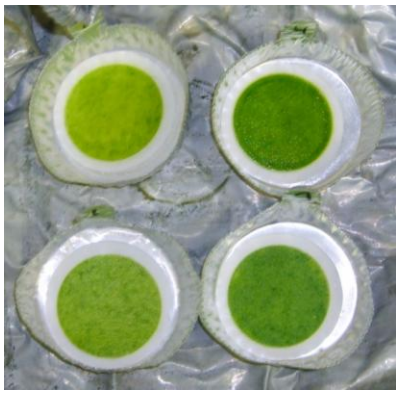


Figure 3.4: Four 10 ml algae samples on Whatman GF/C filters. The samples have been placed onto an aluminum pan for drying in the 65ºC furnace. The two samples on the left are more yellow in color, a typical appearance for nitrate-limited cultures (Rodolfi et al. 2009; Msanne et al. 2012).

A detailed protocol is provided in Appendix A. Dry biomass measurements were for algae samples collected at four points for each of the eight algae cultures grown: when the culture was inoculated, during the early exponential phase, when the culture was harvested for RNA, and when the culture had reached the stationary growth phase.

3.3.3 Nutrient Analysis

Ion chromatography was used to determine the concentration of nitrate remaining in the culture medium during the algal growth period. Immediately following sample collection, approximately 1.5 ml of algae culture was filtered through a 0.45 µm syringe filter and frozen at -20ºC until analysis, which was performed between 18 and 40 days after freezing the samples. Freezing has been verified as a suitable method for the long-term preservation of samples for nitrate analysis (Avanzino and Kennedy 1993). The filtering removed particles and biomass that might clog the ion chromatography column, and freezing preserved the sample and prevented further nitrate consumption due to bacterial growth. Before analysis, the samples were thawed completely and vortexed, and 1:20 dilutions of each sample were prepared in 5 ml ion chromatography vials with filter caps (Dionex, Sunnyvale, CA). Analysis was performed using a IC25 ion chromatograph (Dionex, Sunnyvale, CA), using Dionex Seven Anion Ion Standard II to prepare standard solutions and Dionex AS14A Anion Eluent. For quality control, one duplicate sample and one sample spiked with 100 µl of Seven Ion Standard II were run every eight samples, and two check standards and two anion standards were run every sixteen samples. Sample peaks were analyzed using the Dionex PeakNet 6 software package. Ion chromatography analysis for nitrate levels in the media was conducted using media samples collected at four points for each of the eight algae cultures grown: when the culture was inoculated, during the early exponential phase, when the culture was harvested for RNA, and when the culture had reached the stationary phase of growth.

3.3.4 Flow Cytometry

Flow cytometry was used to count the algae cells and analyze the size, internal complexity, and pigment profile of the cells. All flow cytometry measurements were performed using an BD Accuri C6 flow cytometer (San Jose, California) with a CSampler autosampler. Data were collected and analyzed using the CFlow software package (BD Accuri, San Jose, California). Algae samples were vortexed to break up cell clumps and shaken between the injection of each sample to suspend the cells before sample analysis occured. If algae samples were sufficiently dense to exceed 10,000 events per second in the flow cytometer, the maximum number of events the device can analyze per second, the samples were diluted five times using deionized, 0.2 µm-filtered water. Estimated cell counts were taken based on the number of events per volume, with gates set at a forward scatter threshold of 80,000 to eliminate small particles in the culture media and a 675±12.5 nm filter threshold of 10,000 to exclude cells without chlorophyll fluorescence. The use of the flow cytometer filters to identify and screen cell populations based on phytoplankton pigments has been documented for the C6 device (Adolf et al. 2011). Flow cytometry analysis was conducted using algae samples collected at four points for each of the eight algae cultures grown: when the culture was inoculated, during the early exponential phase, when the culture was harvested for RNA, and when the culture had reached the stationary phase of growth.

3.3.5 Lipid Analysis

Lipids were extracted from each culture using algae samples collected at two points for each of the eight algae cultures grown: when the culture was harvested for RNA and when the culture had reached the stationary phase of growth. Lipids were extracted using a modified Folch protocol (Folch et al. 1957), as described in detail in Appendix A. Nonetheless, lipid analysis using Soxhlet extraction has consistently yielded higher lipid content for experiments on

co-culture samples (unpublished data). The extractions were performed in triplicate for each algae sample.

3.4 Extraction of Total RNA

3.4.1 Preservation and Storage of Samples for RNA Extraction

When the cultures had each reached the late exponential phase, 3 L of each culture were collected in 500 ml centrifuge tubes and placed in ice. Algae was centrifuged in a Beckman J-25 centrifuge at 6000x*g* for 5 minutes at 4ºC, as described by Blanc et al. 2010 for the *Chlorella variabilis* NC64A samples used to prepare a cDNA library for *Chlorella*. Following centrifugation, the supernatant was decanted, taking care not to lose the collected algae. The resulting algae slurry was poured into 50 ml conical bottom conical bottom polypropylene plug-seal centrifuge tubes, with 5 ml of slurry per tube. The algae were flash-frozen using liquid nitrogen and stored on dry ice and ultimately stored in a -80ºC freezer.

3.4.2 RNA Extraction

Total RNA was extracted from the frozen culture samples combining two procedures used for RNA extraction from green algae (Machida et al. 2008; Thanh et al. 2009). Frozen samples were removed from the -80ºC freezer. To each 5 ml frozen sample, 1 ml of beta-mercaptoethanol; 5 ml of extraction buffer (Thanh et al. 2009); 5 ml of a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1 v/v/v, pH 5.2); and 5 ml of 0.5 mm diameter acid-washed glass beads (personal correspondence, Ken-Ichi Honjoh) were added. The extraction buffer (Thanh et al. 2009) consisted of 100 mM Tris-HCl (pH 8.2), 1.4 M NaCl, 20 mM EDTA (pH 8.0), and 2% w/v CTAB. The ionic detergent cetyltrimethylammounium bromide (CTAB) is often employed in nucleic acid extraction procedures; CTAB complexes with polysaccharides in solutions with an ionic strength exceeding 0.7 M and is subsequently removed in the phenol:chloroform extraction procedure (Sambrook and Russell 2001). Samples were

homogenized by vortexing with glass beads for a total of 10 minutes, using ten cycles of 1 minute vortexing at maximum speed followed by 1 minute on ice (personal correspondence, Ken-Ichi Honjoh). Following homogenization, the lysate was transferred to a 50 ml Nalgene Oak Ridge 3119 polypropylene copolymer centrifuge tube (Thermo Fisher Scientific, Rochester, NY) by polyethylene transfer pipet. For maximal recovery of the cell lysate, the glass beads were washed with 2 ml of the phenol:chloroform:isoamyl alcohol solution followed by 2 ml of the extraction buffer, with these solutions being added to the same 3119 tube.

The remainder of the extraction procedure is modified from an RNA extraction procedure used for *Chlorella vulgaris* (Machida et al. 2008). First, the cell lysate undergoes three phenol:chloroform extractions. The sample lysate in the 3119 tube was centrifuged at 2300x*g* for 10 minutes at 4ºC. Two additional phenol:chloroform:isoamyl extractions were performed as follows: the aqueous phase was transferred to a new 3119 tube, 5 ml of phenol:chloroform:isoamyl alcohol was added, and the tube was vortexed for 1 minute then centrifuged for at 2300x*g* for 10 minutes at 4ºC. RNA extraction tubes for the Chl culture and the CC culture before centrifugation and after each of the 3 centrifugations is shown in Figure 3.5.

After the third centrifugation, the aqueous layer was collected into a new 3119 tube for nucleic acid precipitation. Five-hundredths of a volume of 5 M NaCl solution was added to the 3119 tube with the aqueous phase, followed by the addition of 2.5 volumes of absolute ethanol. Nucleic acid precipitation occurred for 1 h at -20ºC, and then the nucleic acid was pelleted by centrifugation at 3000x*g* for 40 minutes at 4ºC.

Finally, the RNA was precipitated using lithium chloride, which is commonly used in total RNA extraction procedures to separate RNA from DNA, proteins, and polysaccharies

(Thanh et al. 2009). The solution was decanted, leaving the nucleic acid pellet. The pellet was dissolved in 1 ml of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.5). Two-hundred ml of a 12 M LiCl were added to achieve a LiCl concentration of 2 M, and the sample was allowed to precipitate overnight at 4ºC. Finally, total RNA was pelleted by centrifugation at 20,000x*g* for 30 minutes at 4ºC. The RNA pellet was washed twice with ice-cold 70% v/v ethanol, air dried to remove residual ethanol, and dissolved in 400 µl of TE buffer.

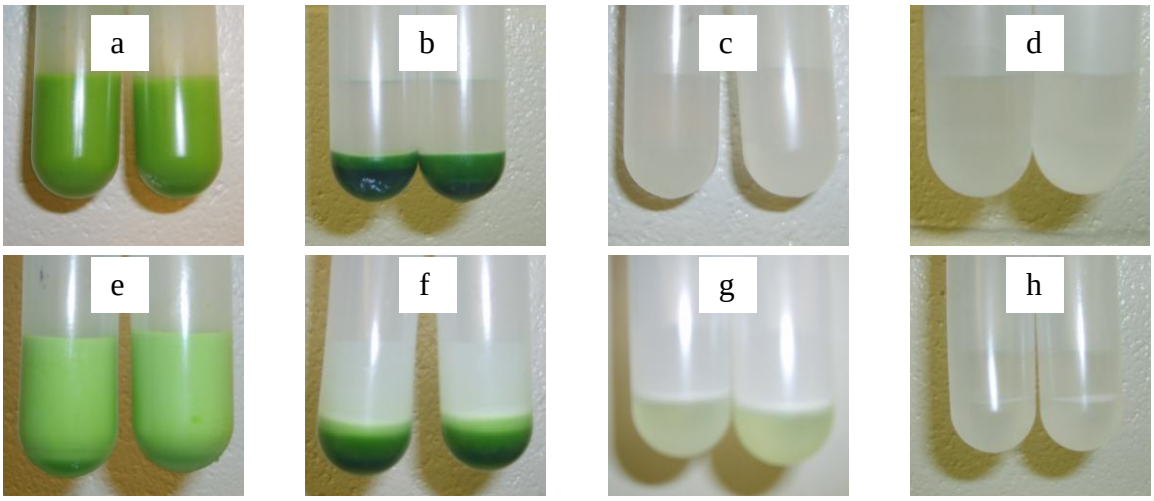


Figure 3.5: Photographs of RNA extraction tubes showing phenol:chloroform extraction before centrifugation and after first (b,f), second (c,g), and third centrifugations (d,h) for a Chl culture (a-d) and a CC culture (e-h). Note that the CC extraction shows a slight interphase even after the third centrifugation (h), whereas the Chl extraction shows little interphase even after the second centrifugation (c).

3.4.3 Preparation of RNA for cDNA Synthesis

Total RNA samples were analyzed to determine concentration and test for impurities using UV spectroscopy. Absorbances were taken at 260 nm, 280 nm, and 230 nm. The A_{260}/A_{280} and A_{260}/A_{230} ratios were computed to check for protein and polysaccharide contamination, respectively. Deoxyribonuclease (DNase) treatment was performed using the

TURBO DNA-*free*™ kit (Ambion, Grand Island, New York) according to the manufacturer's protocol. The RNA samples were further purified up and concentrated using RNA Clean & Concentrator™-5 columns (Zymo Research, Irvine, California) according to the manufacturer's protocol. Finally, the quality of the samples was verified using reverse transcription, which was performed using 400 ng of each RNA sample and an oligo$(dT)_{18}$ primer using the SMARTScribe™ Reverse Transcriptase enzyme (Clontech, Mountain View, California) according to the manufacturer's protocol. Detailed protocols are provided in Appendix A.

3.5 cDNA Synthesis and Amplification

Total RNA samples were prepared for suppression subtractive hybridization through cDNA synthesis and amplification. Samples were removed from the -80ºC freezer and placed on ice. Complementary DNA (cDNA) was synthesized from total RNA using the SMARTer™ PCR cDNA Synthesis Kit (Clontech, Mountain View, California) according to the manufacturer's protocol, following a version of the protocol specific to samples later used in the PCR-Select™ cDNA Subtraction Kit (Clontech, Mountain View, California). The protocol for the SMARTer kit consisted of the following steps: first-strand cDNA synthesis, cDNA amplification by PCR, column chromatography, RsaI digestion, and purification of digested DNA. The SMARTer kit only amplifies poly-(A)+ RNA, which includes most eukaryotic messenger RNA (mRNA). Since *Chlorella vulgaris* was the only eukaryote present in the culture, the only cDNA used in downstream procedures would be made from *Chlorella vulgaris* mRNA. RNA from the *Leptolyngbya* sp., any bacteria in the cultures, or other types of RNA, would be amplified. This aspect of the procedure allowed the use of suppression subtractive hybridization on total RNA extracted from cultures whose only eukaryote was *Chlorella vulgaris*, despite the presence of prokaryotes, the bacteria and cyanobacteria. Detailed experimental protocols for the cDNA synthesis and amplification are provided in Appendix A.

3.6 Suppression Subtractive Hybridization

3.6.1 Introduction to Suppression Subtractive Hybridization

Suppression subtractive hybridization (SSH) is a technique used to isolate and exponentially amplify genes that are differentially expressed in one pool of DNA compared to another. The following image (Figure 3.6) is commonly used to demonstrate the SSH process, which is explained in detail in the PCR-Select cDNA Subtraction Kit manual (Clontech Laboratories 2011a) as well as several key references on the technique (Diatchenko et al. 1996; Akopyants et al. 1998).

3.6.2 Suppression Subtractive Hybridization: Description of Subtracted cDNA Libraries

A total of 8 subtractive hybridizations were performed, generating 8 subtracted cDNA libraries.

Table 3.5: Summary of Subtracted cDNA Libraries

Abbreviated Name of Library	Irradiance Level of Culture	Nitrate Level of Culture (% of Bold's Basal Medium)	Description of cDNA in the Library
Up1vs5	180 µmol/m^2-s	50%	Genes upregulated in *Chlorella vulgaris* in the Chl culture
Up5vs1	180 µmol/m^2-s	50%	Genes upregulated in *Chlorella vulgaris* in the CC culture
Up2vs6	180 µmol/m^2-s	100%	Genes upregulated in *Chlorella vulgaris* in the Chl culture
Up6vs2	180 µmol/m^2-s	100%	Genes upregulated in *Chlorella vulgaris* in the CC culture
Up3vs7	400 µmol/m^2-s	50%	Genes upregulated in *Chlorella vulgaris* in the Chl culture
Up7vs3	400 µmol/m^2-s	50%	Genes upregulated in *Chlorella vulgaris* in the CC culture
Up4vs8	400 µmol/m^2-s	100%	Genes upregulated in *Chlorella vulgaris* in the Chl culture
Up8vs4	400 µmol/m^2-s	100%	Genes upregulated in *Chlorella vulgaris* in the CC culture

Four subtractive hybridizations were done to identify genes upregulated in the CC cultues compared to the Chl cultures, and four subtractive hybridizations were done to identify genes

upregulated in the Chl cultures compared to the CC cultures. The subtractive libraries are listed in Table 3.5.

In each case, the tester pool of cDNA was from the population for which upregulated genes were identified, and the driver cDNA pool was from the other population grown at the same irradiance and nitrate levels. For example, in the up1vs5 library, cDNA from *Chlorella vulgaris* in the Chl grown at an irradance of 180 µmol/m^2-s and 50% the nitrate level of Bold's Basal Medium served as the tester, and cDNA from the *Chlorella vulgaris* in the CC culture grown at the same irradiance and nitrate levels served as the driver.

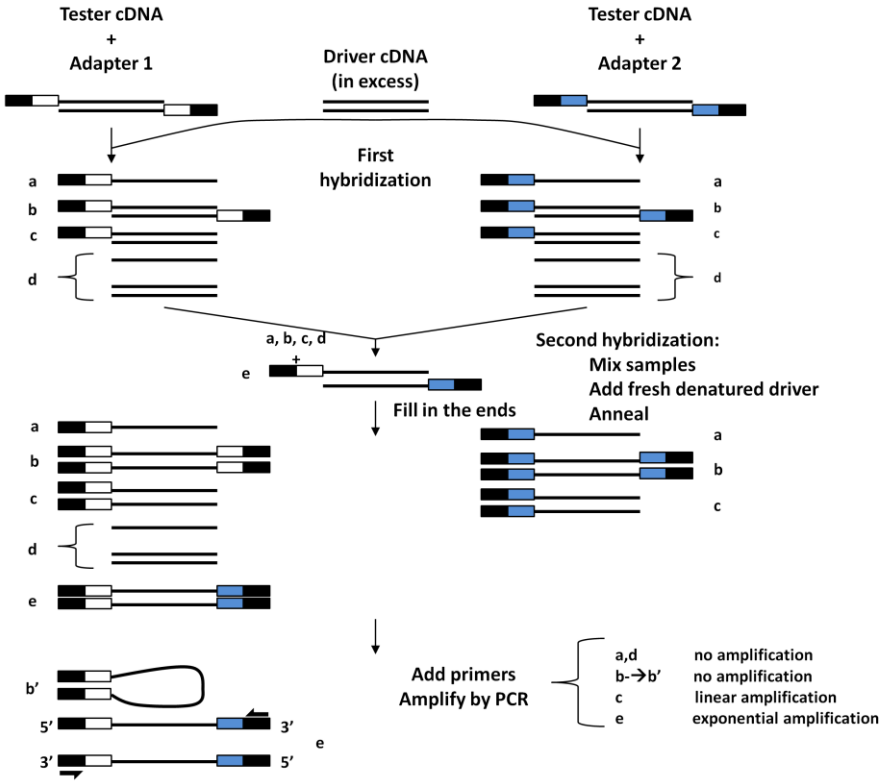


Figure 3.6: Schematic of Suppression Subtraction Hybridization, as performed using the Clontech PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories 2011a). Lines represent cDNA following digestion by RsaI. Black boxes adjoined to white boxes represent Adapter 1; black boxes adjoined to shaded boxes represent Adapter 2R. The black boxes correspond to PCR Primer 1, the white boxes to Nested PCR Primer 1, and the shaded boxes to Nested PCR Primer 2R.

3.6.3 Suppression Subtractive Hybridization: Experimental Method

Suppression subtractive hybridization (SSH) was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech, Mountain View, California) according to the manufacturer's protocol. As described previously, the entire suppression subtractive hybridization process consists of the following steps: first-strand cDNA synthesis, second-strand cDNA synthesis, RsaI digestion, adapter ligation, first hybridization, second hybridization, and PCR amplification. Since cDNA synthesis and RsaI digestion had been performed using the SMARTer kit, the first step performed using the PCR-Select™ cDNA Subtraction Kit was adapter ligation. A diagram showing one a pair of subtractive hybridizations, those used to generate the aforementioned up1vs5 and up5vs1 subtracted libraries, is provided in Figure 3.7 to facilitate understanding of the process. The other 6 hybridizations follow the same pattern.

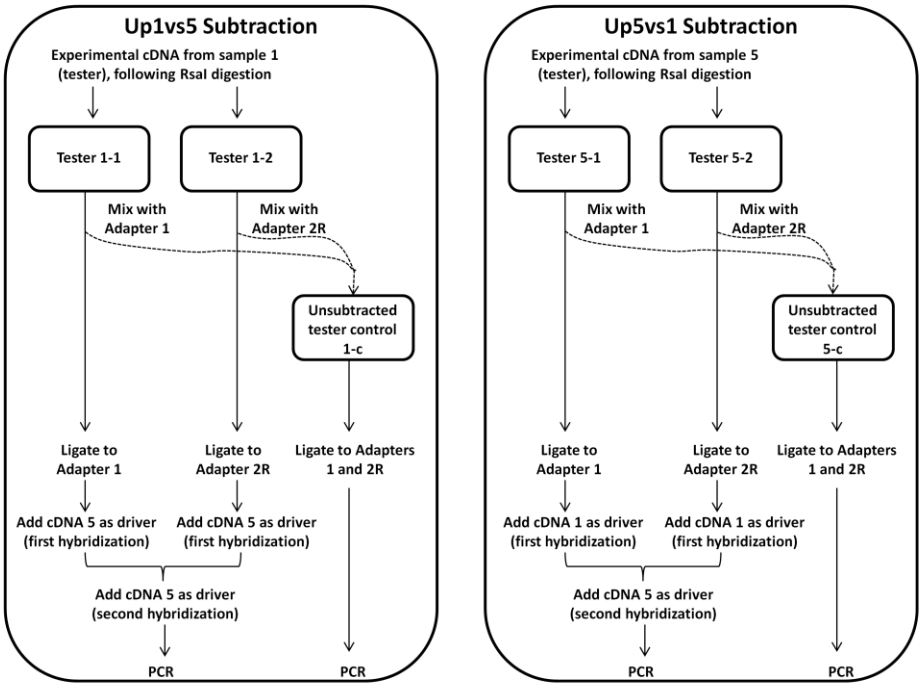


Figure 3.7: Diagram of adapter ligation, first hybridization, and second hybridization processes involved in the generation of subtracted cDNA libraries up1vs5 and up5vs1. Adapted from PCR-Select User Manual (Clontech Laboratories 2011a).

Detailed experimental protocols for the suppression subtraction hybridizations are provided in Appendix A.

3.7 Preparation of SSH Products for DNA Sequencing

3.7.1 Introduction to *E. coli* Subcloning

Following secondary PCR in the SSH process, the PCR products, representing expressed sequence tags (ESTs) of differentially expressed genes, were prepared for subcloning into *E. coli*. The process of subcloning accomplished two key goals. First, DNA sequencing requires that each sample prepared for sequencing contain copies of only one sequence. Second, each unique EST needs to be present in an ideal amount for successful sequencing. Both goals are accomplished through *E. coli* subcloning (Lodish et al. 1995), which was performed using the pGEM®-T Easy Vector System with JM109 competent *E. coli* cells (Promega, Madison, Wisconsin) according to the manufacturer's protocol.

The process of subcloning consists of four steps: digestion of DNA into fragments, ligation into a cloning vector, transformation of the cloning vector into the cells, and screening for cells that contain the vector with the insert (Padmanabhan et al. 2011). During the SSH process, the DNA has already been digested. The remaining three processes were performed using the pGEM-T Easy Vector System (Promega, Madison, Wisconsin). Screening for the cells was performed using ampicillin (to screen for cells containing the vector) and blue/white selection (to verify that cells containing the vector had the vector with the insert) (Promega 2009).

The resulting white colonies from the blue/white selection each contain one DNA insert. By selecting these colonies and growing each colony separately, the inserted DNA can be copied until a quantity sufficient for DNA sequencing is obtained. In this way, *E. coli* subcloning accomplishes both goals, separating the pool of DNA sequences so that each sequence is isolated

from a mixture of DNA and a quantity of each sequence sufficiently large for DNA sequencing is obtained.

3.7.2 Preparation of SSH Products for Sequencing and DNA Sequencing

The PCR products were purified using the NucleoTraP®CR silica matrix-based purification system (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Adenylation was performed using Taq Full Hot Start DNA Polymerase (Clontech, Mountain View, California), which was performed according to a protocol in the Technical Manual for the pGEM®-T and pGEM®-T Easy Vector Systems (Promega, Madison, Wisconsin).

Following adenylation, the SSH products were ligated into the pGEM®-T Easy Vector (Promega, Madison, Wisconsin) and transformed into JM109 E. coli cells according to the manufacturer's protocol. Transformed *E. coli* were grown on LB agar plates with ampicillin/ IPTG/X-Gal. Colonies were grown in deep-well blocks in an incubated shaker for 20 hours, and the resulting E. coli colonies were submitted for sequencing. Extraction of the plasmids containing the DNA and the DNA sequencing were performed by Functional Biosciences (Madison, Wisconsin). DNA sequencing was performed using dye-terminator method on the 3730xl DNA Sequencer with 50 cm arrays (Applied Biosystems, Carlsbad, California). The T7 Promoter sequencing primer was used.

3.8 Quantitative PCR of Selected Genes

Following sequencing of the DNA from the subtracted libraries, quantitative PCR (qPCR) was performed on selected genes. Quantitative PCR was performed for two purposes. First, by quantitatively measuring the levels of gene expression, false positives among the genes tested in the subtracted libraries would be identified. Second, while suppression subtractive hybridization yields a pool of putatively differentially expressed genes, due to the normalization

that occurs in SSH, no information regarding the level of upregulation of the genes is known. Quantitative PCR provided quantitative data regarding the levels of upregulation.

3.8.1 Quantitative PCR: Background of Technique

Quantitative polymerase chain reaction (qPCR), is a fluorescence-based technique which allows for the accurate quantification of levels of nucleic acid (Bustin et al. 2009). Quantitative PCR is also commonly referred to as real-time PCR (RT-PCR) but herein shall only be referred to as qPCR to prevent ambiguity with reverse-transcription polymerase chain reaction (which shares the acronym RT-PCR), which refers to the reverse transcription of RNA into cDNA followed by the PCR amplification of the cDNA (Farrell 2009). qPCR involves the measurement of the amount of nucleic acid at the end of the elongation step of each PCR cycle through the use of fluorescence. The most commonly used fluorescence source is the dye SYBR Green, which fluoresces when bound to double-stranded DNA. As the template DNA is amplified in the qPCR process, the fluorescence resulting from SYBR Green binding to the amplicon increases in proportion to the amount of amplified product (Farrell 2009).

The amplification of a DNA template through PCR is commonly described by the equation $N = N_0 (1 + eff)^n$, in which N represents the number of copies of the DNA amplicon at the end of PCR, N_0 represents the number of copies before PCR, eff represents the reaction efficiency, and n represents the number of PCR cycles (Farrell 2009). Efficiency is a decimal between 0 and 1 and provides a measurement of the level of amplification. An efficiency of 1 means that in each PCR cycle, the number of copies of the amplicon doubles. Nonetheless, this equation only holds for the early cycles of PCR. In later cycles of PCR, a variety of factors reduce the efficiency of PCR. First, the activity of the polymerase is reduced with exposure to heat during successive denaturing steps in each cycle of PCR, and the amount of polymerase available to perform the reaction is fixed. Second, the concentrations of the reagents for the PCR

reaction, dNTPs (deoxynucleotide triphosphates) and primers, decrease as they are incorporated into the amplified DNA. Therefore, the exponential nature of PCR, as described in the equation, only holds in the early cycles of the process. The later cycles a linear phase and a plateau phase. The exponential phase, linear phase, and plateau phase of PCR are shown in Figure 3.8 (Farrell 2009).

The PCR plateau effect is important in understanding qPCR because only when amplification occurs exponentially, according to the aforementioned equation, can accurate quantification of the original amount of the template be performed. By measuring the fluorescence of the amplified DNA during the exponential phase, the quantity of the target DNA in the original sample is known.

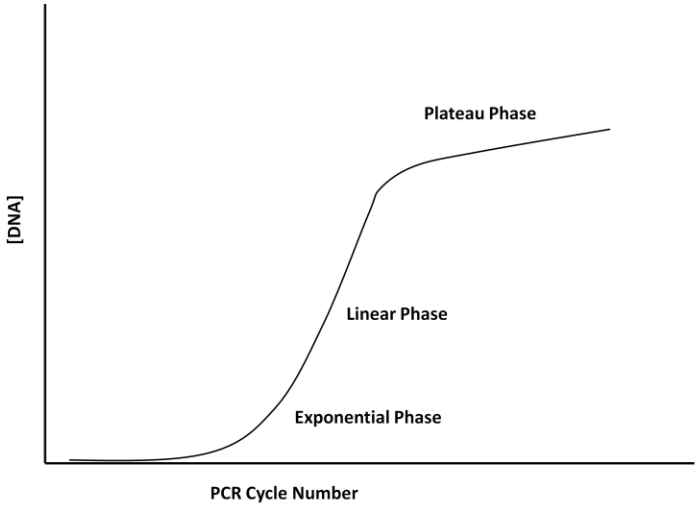


Figure 3.8: The three phases of PCR, demonstrating the PCR plateau effect (Farrell 2009). Exponential amplification occurs only during the exponential phase, after which amplification becomes linear. For proper quantification of template amounts in the original sample, data from the exponential phase must be used.

Quantitative PCR involves two key plots, the amplification plot and the melting curve plot. During the qPCR reaction, the level of fluorescence is measured after the elongation step of each PCR cycle. The plot of fluorescence against amplification cycle is known as the amplification plot (Figure 3.9). The solid line represents the fluorescence threshold, the point at which the fluorescence signal becomes detectable above background noise. The horizontal axis coordinate at which the amplification curve intercepts the threshold line is the quantification cycle value (C_q), which is also called the cycle threshold (C_t). Note that the C_q value is determined during the exponential phase of PCR, during which accurate quantification can occur.

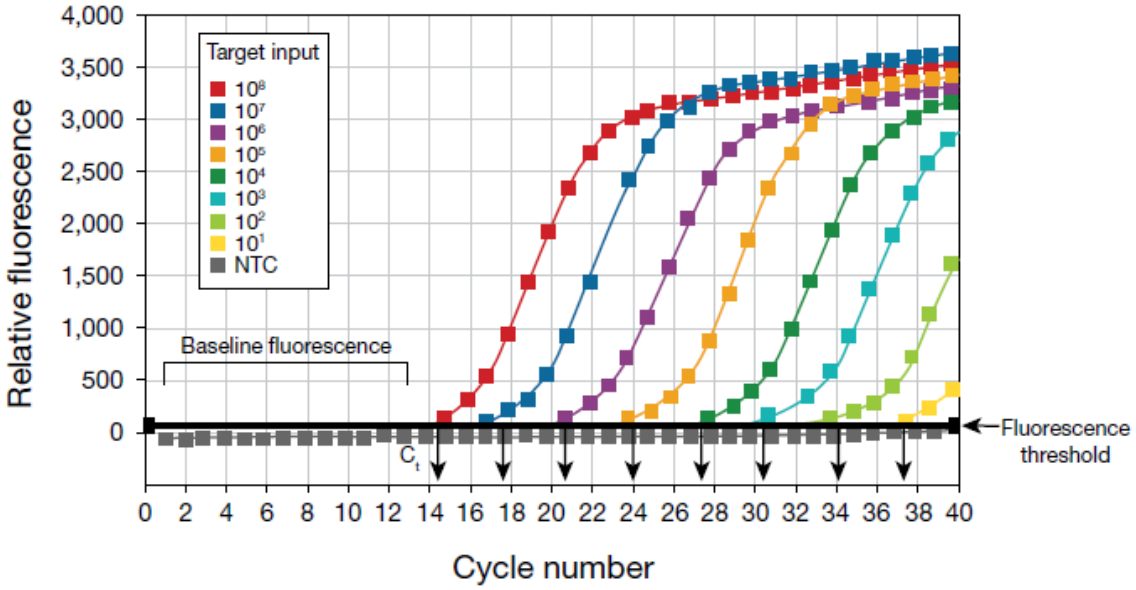


Figure 3.9: An example of an amplification plot, showing amplification curves for serial dilutions of a DNA template (Invitrogen).

Since SYBR Green will bind to any double-stranded DNA molecule, for accurate quantification of a specific DNA template, it must be known that the fluorescence signal represents only the desired template. Melting curve analysis, in which the melting curve plot is

generated, is used to determine that only the specific product is amplified. An example melting curve plot is shown in Figure 3.10.

Following qPCR, a melting curve is generated by slowly raising the temperature of the reaction mixture and measuring fluorescence. As double-stranded DNA is melted and separated into single strands, SYBR Green can no longer bind, and the fluorescence decreases (Figure 3.10). A single, pronounced peak in the first-derivative plot of fluorescence (Figure 3.11) against temperature indicates that only a single product has been amplified in the qPCR reaction.

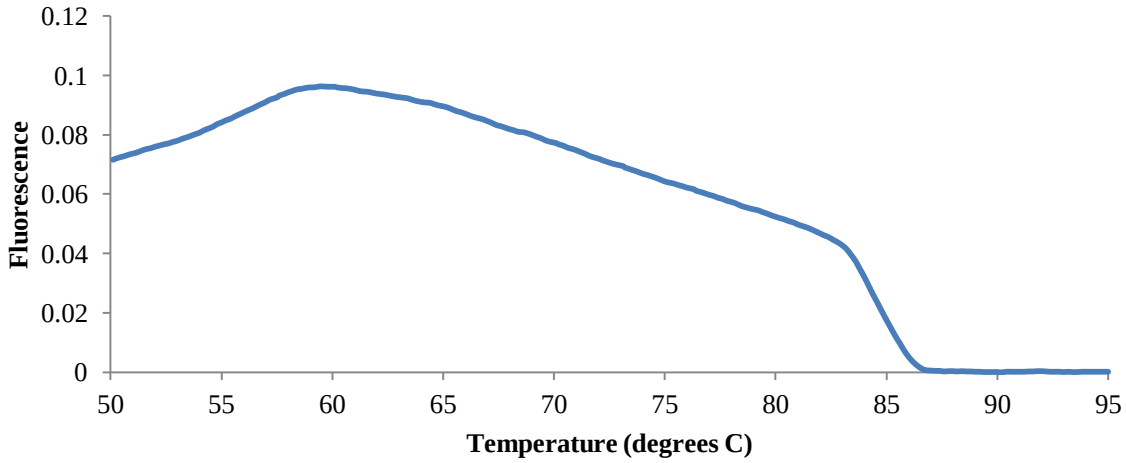


Figure 3.10: A melting curve plot of fluorescence. The fluorescence level shows a sharp decrease around the amplicon melting temperature of 84°C. (image source: unpublished data)

The presence of multiple peaks indicates that multiple products have been amplified, and fluorescence data does not reflect accurate quantification of only the desired product but rather measures the amount of both the desired and the undesired products.

Quantification in qPCR can be either absolute or relative. In absolute quantitation, a standard curve is prepared with from a sample with a known number of copies of the desired DNA sequence. The standard curve is a linear regression of the plot of the quantification cycle

versus the log of copy number of the DNA template is taken. The C_q is determined for an unknown sample, and then the copy number for the DNA target is determined using the standard curve (Farrell 2009).

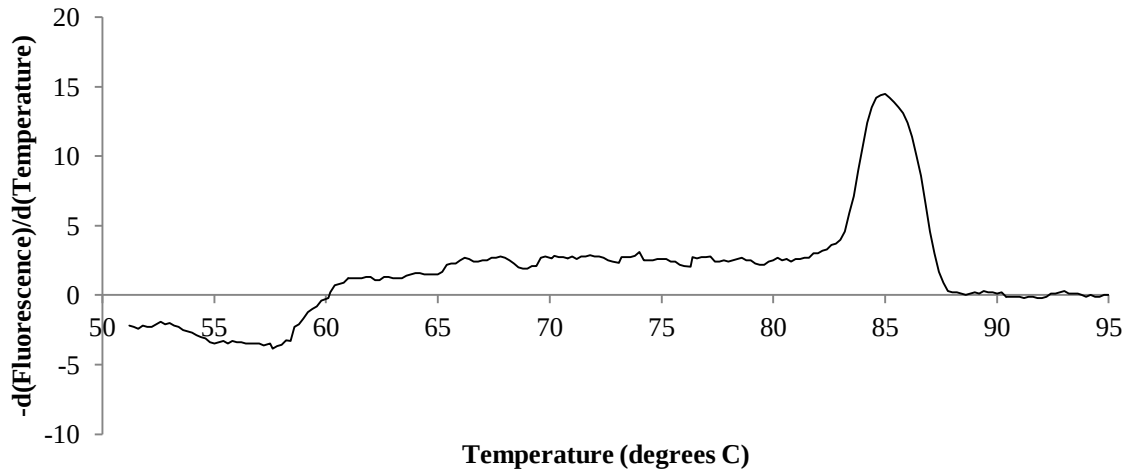


Figure 3.11: A melting curve plot of the negative first derivative of fluorescence. A single peak shows that the qPCR reaction has only amplified one product.

In relative quantification, the gene of interest is compared to a reference gene or the same gene in a control sample. The C_q values of both the gene of interest and the reference gene are used to determine the relative levels of expression of the gene of interest compared to the reference gene. One key advantage of relative quantification is that the copy number of the desired DNA template does not need to be known.

In summary, qPCR is a technique that can be used to determine the absolute level of a particular DNA template or the relative level of that template compared to another template. The technique accomplished quantification by using a fluorescent dye that generates a signal proportional to the amount of double-stranded DNA present in the qPCR reaction at a particular time. This fluorescence signal is measured after the elongation step of each PCR cycle, and the

resulting quantification cycle value (C_q) provides quantitative information regarding the amount of the desired template originally present in the sample. Finally, the melting curve is generated to gauge the specificity of the qPCR amplification, making sure that the fluorescence signal is generated only from the desired DNA template.

3.8.2 Preparation of Total RNA Sample for Quantitative PCR

Ten µg of total RNA for each sample was prepared for use in qPCR reactions. Deoxyribonuclease (DNase) treatment was performed using the TURBO DNA-*free*™ kit (Ambion, Grand Island, New York) according to the manufacturer's protocol. The RNA samples were further purified up and concentrated using RNA Clean & Concentrator™-5 columns (Zymo Research, Irvine, California) according to the manufacturer's protocol. Reverse transcription was performed using the SMARTScribe™ Reverse Transcriptase enzyme (Clontech, Mountain View, California) according to the manufacturer's protocol, using 3 µg of total RNA in a 40 µl reaction with an oligo$(dT)_{18}$ primer. The reverse transcribed products were diluted by the addition of 80 µl of water to each reaction tube. Detailed protocols are provided in Appendix A.

3.8.3 Quantitative PCR: Experimental Protocol

Quantitative PCR was performed using the diluted reverse transcription products from the total RNA for each of the eight samples. For each reaction, 1 µl of template containing the reverse transcription products of 25 ng of total RNA was used. Reactions were performed using the MiniOpticon™ Real-Time PCR Detection System (Bio-Rad, Hercules, California) in GeneMate 8-Strip UltraFlux white PCR tubes with optically clear flat caps (Catalog Number T-3224-1; BioExpress, Kaysville, Utah). Thermal cycling conditions were 3 minutes at 95°C to activate the enzyme and 40 cycles of 3 seconds at 95°C, 20 seconds at 60°C, and 2 seconds at

72°C followed by a plate read to measure fluorescence. Immediately following thermal cycling, a melting curve was generated by heating the reactions to 95°C for 1 minute, dropping the temperature to 50°C, and raising the temperature from 50°C to 95°C in 0.2°C increments, collecting fluorescence at each step. Data was analyzed with the MJ Opticon™ Monitor software (3.1.32; Bio-Rad, Hercules, California).

Statistical analysis on gene expression levels was performed using SAS® (9.3; SAS Institute Inc., Cary, North Carolina) using the quantification cycle values. ANOVA was performed with LSD post-hoc test and Tukey adjustment for multiple comparisons to test pairwise significance of gene expression levels for cultures grown at the same irradiance and nitrate level. To test the effects of irradiance or nitrate level across the 4 Chl, 4 CC, or 8 cultures, the non-parametric Wilcoxon rank-sum test was used because there were two levels of each effect. Finally, to test the effects of expression level across all 8 cultures, the Kruskal-Wallis test was used because there were 8 groups, one for each culture. The use of non-parametric tests, the Wilcoxon rank-sum test and the Kruskal-Wallis test, did not require the assumption of homogeneity of variance as does ANOVA.

Nomenclature used for the gene expression results for the 8 cultures is presented in Table 3.6.

Table 3.6: Nomenclature Used for qPCR Gene Expression Results

Nomenclature	Description
Chl	Refers to the *Chlorella vulgaris* monoculture
CC	Refers to the *Chlorella vulgaris* in the co-culture
180L50N	Refers to cultures grown at a scalar irradiance of 180 µmol/m^2-sec and a starting nitrate concentration at 50% of that of Bold's Basal Medium
180L100N	Refers to cultures grown at a scalar irradiance of 180 µmol/m^2-sec and a starting nitrate concentration at 100% of that of Bold's Basal Medium
400L50N	Refers to cultures grown at a scalar irradiance of 400 µmol/m^2-sec and a starting nitrate concentration at 50% of that of Bold's Basal Medium
400L100N	Refers to cultures grown at a scalar irradiance of 400 µmol/m^2-sec and a starting nitrate concentration at 100% of that of Bold's Basal Medium

Gene expression levels were normalized according to the amount of total RNA used per qPCR reaction (Bustin 2000; Bustin 2002; Bergkvist et al. 2008). Data was analyzed using the efficiency-corrected model (Pfaffl 2001), and the efficiencies for each gene are provided in Appendix C. Relative quantities of cDNA transcripts corresponding to the gene for the 8 samples were normalized to the lowest expression among the 8 samples. Statistical analysis was performed to determine if the gene expression levels between the Chl and CC grown at the same conditions was significant. Additionally, the statistical significance of the effects of irradiance and nitrate across the 8 samples were analyzed to determine if irradiance and/or nitrate affected gene expression levels.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Algae Growth

4.1.1 Growth Curves

Growth curves for the cultures, measured as the absorbance at 664 nm in a poly(methyl methacrylate) spectrophotometer cuvette with a path length of 1 cm, are provided in Figure 4.1 for the CC and Figure 4.2 for the Chl cultures. Growth curves were used to determine when the cultures were in the late exponential phase, when cultures were collected for RNA.

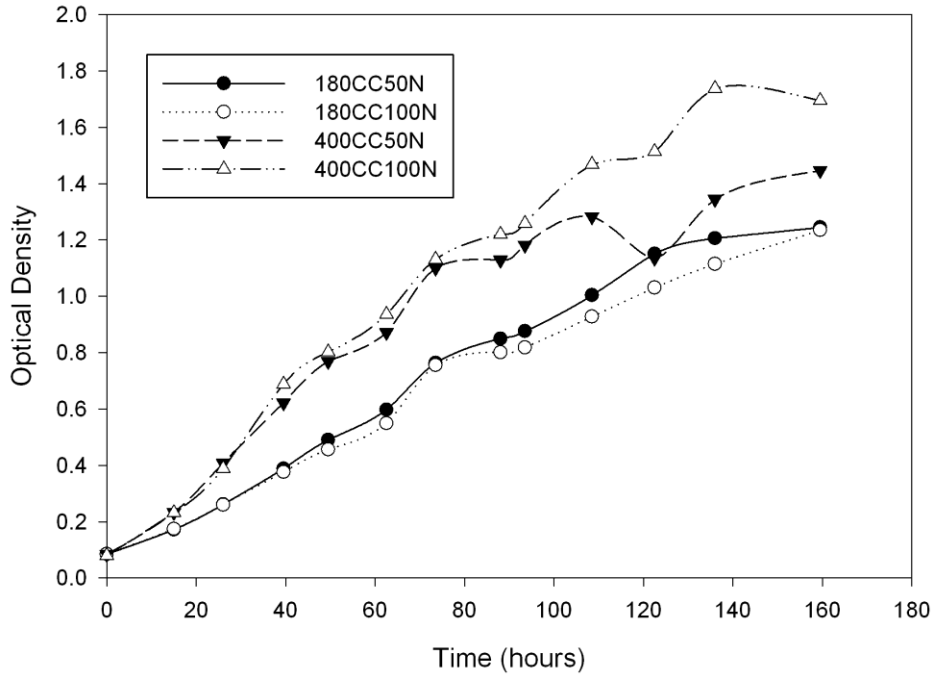


Figure 4.1: Growth curves of the CC culture samples at the 4 culture conditions. Optical density measurements represent 664 nm absorbances. RNA was collected from each culture after they had grown for 108.5 hours, and lipids were extracted from cultures that had grown for 159.5 hours.

Algae samples were collected for RNA for the CC and Chl cultures at 108.5 h and 63 h after the inoculation of the cultures, respectively. The cultures were permitted to continue growth into the stationary phase, at which time algae culture samples were collected for Folch lipid extraction.

For the CC and Chl cultures, respectively, cultures for lipid samples were taken at 159.5 h and 114 h after inoculation. Following removal of 3 L of the 5 L of the culture for the samples collected for RNA, the cultures experienced increased growth rates. This phenomenon can be explained due to less light limitation and to more aeration per unit volume. While the incident irradiance at the surface of the algal growth media was the same as before the 3 L of culture was harvested, a shallower depth of algae culture allowed more light to penetrate the culture. Additionally, the rates of aeration and carbon dioxide dosings were maintained at the same levels as for the 5 L culture volume, which means that the algae in the decreased culture volume had more available carbon dioxide and gas exchange than when the culture volume was 5 L.

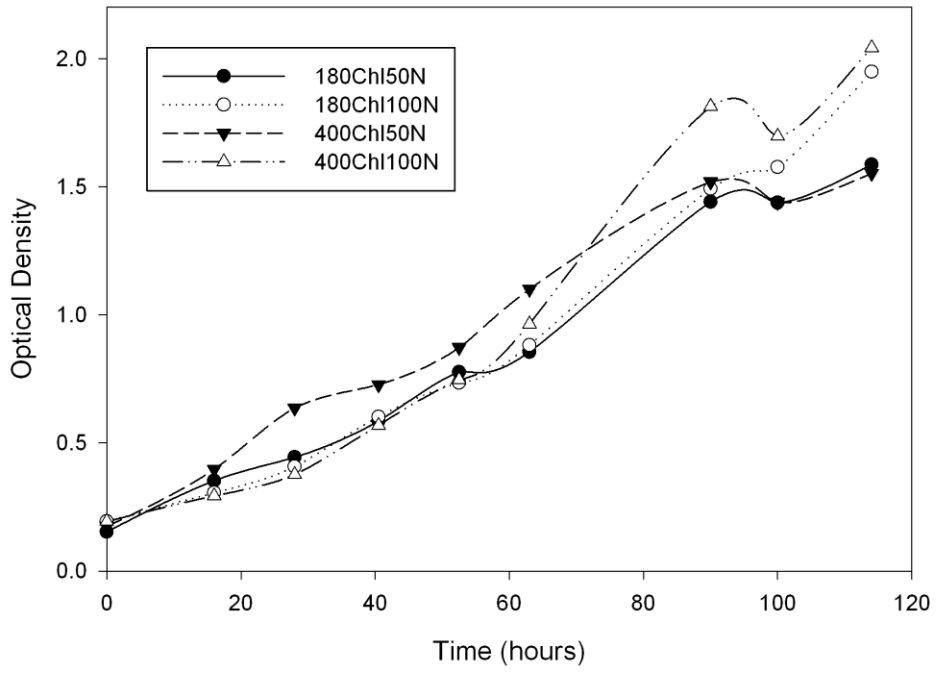


Figure 4.2: Growth curves of the Chl culture samples at the 4 culture conditions. Optical density measurements represent 664 nm absorbances. RNA was collected from each culture after they had grown for 63 hours, and lipids were extracted from cultures that had grown for 114 hours.

4.1.2 Dry Biomass

Dry biomass data for the algae was taken at four points in the growth for each of the 8 cultures. The results for the CC cultures are shown in Table 4.1, and the results for the Chl cultures are shown in Table 4.2.

Table 4.1: Dry Biomass Levels (g/L) for the Co-culture

Culture Condition	Dry Biomass at t=0 h	Dry Biomass at t=49.5 h	Dry Biomass at t=108.5 h	Dry Biomass at t=159.5 h
180CC50N	0.04	0.21	0.50	0.74
180CC100N	0.04	0.20	0.46	0.70
400CC50N	0.04	0.32	0.72	1.08
400CC100N	0.04	0.32	0.80	1.38

Table 4.2: Dry Biomass Levels (g/L) for the *Chlorella* sp. Cultures

Culture Condition	Dry Biomass at t=0 h	Dry Biomass at t=40.5 h	Dry Biomass at t=63 h	Dry Biomass at t=114 h
180Chl50N	0.09	0.23	0.33	0.70
180Chl100N	0.13	0.21	0.31	0.75
400Chl50N	0.10	0.28	0.46	0.71
400Chl100N	0.11	0.21	0.37	1.06

Note that while the dry biomass levels for the two cultures of the 180 µmol/m^2-s irradiance were similar at each of the four time points, the dry biomass levels for the 400 µmol/m^2-s cultures were higher for the 100% nitrate level culture than for the 50% nitrate level culture at 108.5 and 159.5 hours. On the other hand, while the 400 µmol/m^2-s irradiance and 100% nitrate level culture of Chl culture shows the highest biomass at the final sampling time, at the third sampling time, the 400 µmol/m^2-s irradiance and 50% nitrate level culture has the highest dry biomass.

Finally, note that while the 400 µmol/m^2-s irradiance CC cultures have a higher dry biomass at the final sampling time than the Chl culture, all of the 180 µmol/m^2-s irradiance cultures have a similar dry biomass in the stationary phase. The higher biomass at the 400 µmol/m^2-s irradiance level is important as biomass level, along with lipid content, is one key factor in the amount of lipid produced by an algae culture.

4.1.3 Nitrate Analysis

Ion chromatography analysis was performed for the nitrate level in the media for each of the eight cultures at the same four sampling times for which other data was taken. Figure 4.3 shows the nitrate levels in mg/L for the CC cultures, and Figure 4.4 shows the nitrate levels for the Chl cultures. For both the CC and the Chl cultures, nitrate levels followed the same pattern.

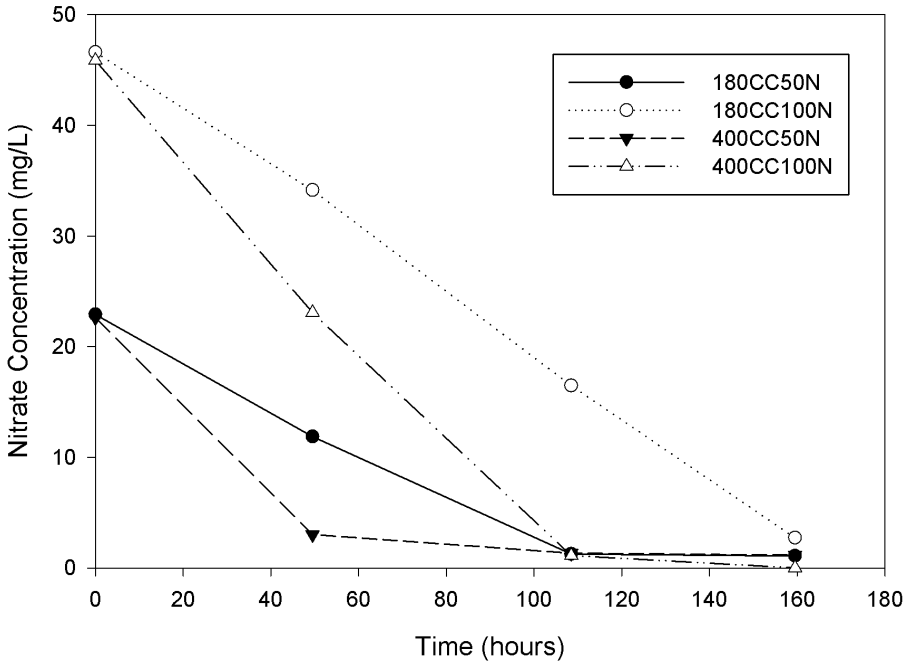


Figure 4.3: Nitrate levels in the CC culture media with respect to time, as determined by ion chromatography. As would be expected, cultures at each irradiance level with 50% the starting nitrate level of Bold's Basal Medium depleted the nitrate in the media more quickly.

For cultures of equal nitrate levels but different irradiances, the nitrate is depleted from the media more quickly in the 50% nitrate level cultures than in the 100% nitrate level cultures, as would be expected. Second, for culture of equal starting nitrate concentration in the media, cultures of 400 µmol/m^2-s scalar irradiance deplete the nitrate in the media faster than do the cultures of 180 µmol/m^2-s scalar irradiance, as would be expected as the culture of higher irradiance has a higher growth rate. Finally, by the time the cultures reach the stationary phase, almost all nitrate in the media has been depleted.

Note that the Chl cultures have a slightly higher starting nitrate concentration in the media than do the co-cultures. This phenomenon is due to residual nitrate from the inoculum. The prescence of more nitrate in the media would prolong the exponential phase as the cultures would grow for a longer period of time before becoming nitrate-limited.

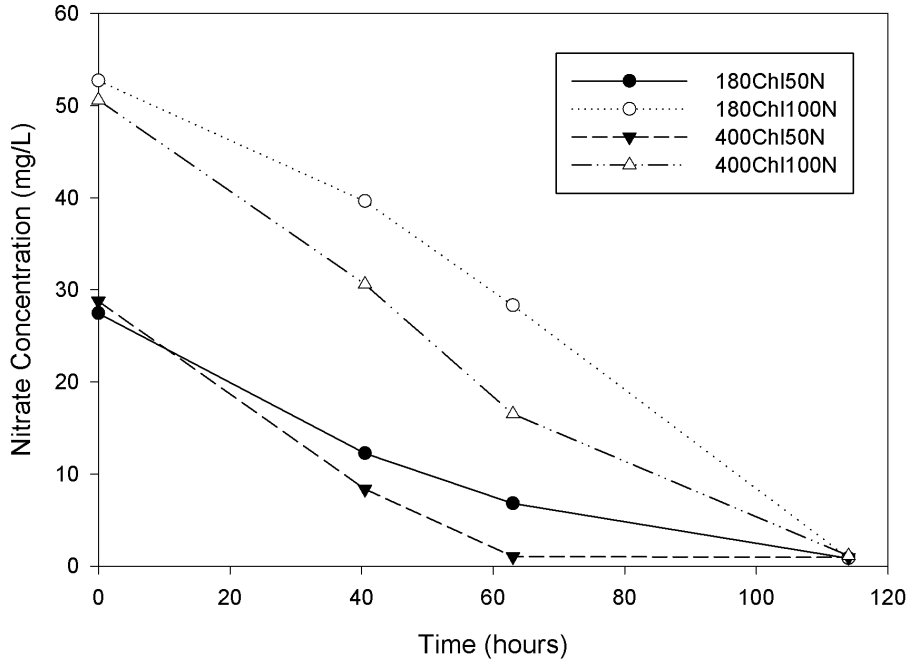


Figure 4.4: Nitrate levels in the Chl culture media with respect to time, as determined by ion chromatography. As would be expected, cultures at each irradiance level with 50% the starting nitrate level of Bold's Basal Medium depleted the nitrate in the media more quickly.

4.1.4 Flow Cytometry

Flow cytometry analysis was conducted using algae samples collected at four points for each of the eight algae cultures grown: when the culture was inoculated, during the early exponential phase, when the culture was harvested for RNA, and when the culture had reached the stationary phase of growth.

Table 4.3: Cell Counts (10^6 cells/ml) for the CC Cultures

Culture Condition	Cell Count at t=0 h	Cell Count at t=49.5 h	Cell Count at t=108.5 h	Cell Count at t=159.5 h
180CC50N	1.51±0.76	8.17±0.21	18.1±0.6	18.5±0.4
180CC100N	1.56±0.78	7.92±0.09	17.7±0.1	24.4±0.3
400CC50N	1.54±0.77	15.1±0.8	23.6±0.2	22.7±0.1
400CC100N	1.52±0.76	16.6±0.2	27.4±0.9	23.1±1.5

Values expressed as mean ± S.D. All analyses were performed in triplicate.

Table 4.4: Cell Counts (10^6 cells/ml) for the Chl Cultures

Culture Condition	Cell Count at t=0 h	Cell Count at t=40.5 h	Cell Count at t=63 h	Cell Count at t=114 h
180Chl50N	4.03±0.08	19.8±0.2	33.2±1.3	128±4
180Chl100N	5.07±0.07	21.7±0.3	33.4±1.1	124±1
400Chl50N	4.14±0.37	20.6±0.6	38.1±1.1	124±5
400Chl100N	4.33±0.07	16.3±0.1	30.8±0.7	125±4

Values expressed as mean ± S.D. All analyses were performed in triplicate.

Cell counts for the CC cultures and the Chl cultures are shown in Tables 4.3 and 4.4, respectively. For the CC cultures, the cell counts at 49.5 hours for the 400 µmol/m^2-s scalar irradiance far exceed those of the 180 µmol/m^2-s scalar irradiance, with this trend holding true at

108.5 hours but not at 159.5 hours, in the stationary phase. On the other hand, the cell counts among the four culture conditions for the Chl cultures are roughly the same at each time when cells were sampled, despite the significantly higher dry biomass of the 400Chl100N culture at 114 h compared to the other three cultures.

Nonetheless, the most significant trend is that the cell counts in the Chl cultures outnumber the cell counts in the CC cultures by nearly five times in the stationary phase ($p<0.0001$, ANOVA with LSD post-hoc test and Tukey adjustment for multiple comparisons), yet the dry biomass for the Chl cultures is similar or lower. This trend is explained by the smaller *Chlorella vulgaris* cells of the Chl cultures as compared to the *Chlorella vulgaris* cells in the CC cultures. While the *Chlorella vulgaris* cells in the CC are fewer in number, they are adequately large to have a similar biomass as the far more numerous *Chlorella vulgaris* cells of the Chl cultures.

The flow cytometry plots show a very different appearance for the monoculture and co-culture. The plots for filter FL4-A (which collects emissions in the range of 675±12.5 nm) versus forward scatter (representing cell size) and FL3-A (which collects emissions of visible light with wavelengths at or exceeding 670 nm) show the clear presence of two populations in the CC culture but only one population in the Chl culture. All 4 plots were taken at the inoculation of the cultures for the culture conditions of 400 μmol/m^2-sec. and 50% the nitrate level of Bold's Basal Medium.

Plots of cell counts vs. filter FL4-A (675±12.5 nm) and side scatter (representing cell complexity) vs. forward scatter also show the presence of the two populations in the CC culture (Figure 4.6). Once again, the *Chlorella vulgaris* cells in the CC culture have a larger average size as compared to the *Chlorella vulgaris* cells in the Chl culture. All 4 plots were taken at the

inoculation of the cultures for the culture conditions of 400 µmol/m^2-sec and 50% the nitrate level of Bold's Basal Medium.

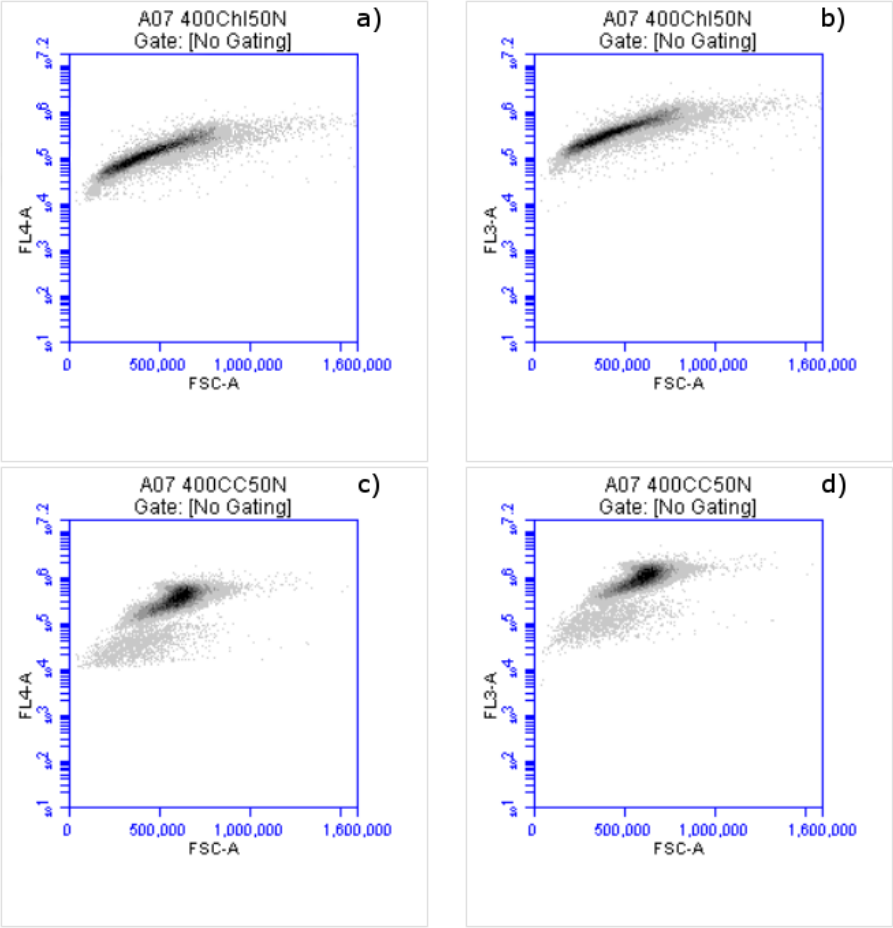


Figure 4.5 :Flow cytometry plots of the Chl culture (a and b) and the CC culture (c and d). Plots a and c show filter FL4-A (675±12.5 nm), which collects fluorescence from chlorophyll-a, plotted against forward scatter, representing cell size. Plots b and d show filter FL3-A (670 nm long-pass), which collects fluorescence from chlorophyll and phycocyanins. Note the presence of the two cell populations in c and d and the larger average size of the *Chlorella vulgaris* population in the co-culture, shown by the dense area of plots c and d, compared to that of the *Chlorella vulgaris* in the Chl culture, show in plots a and b.

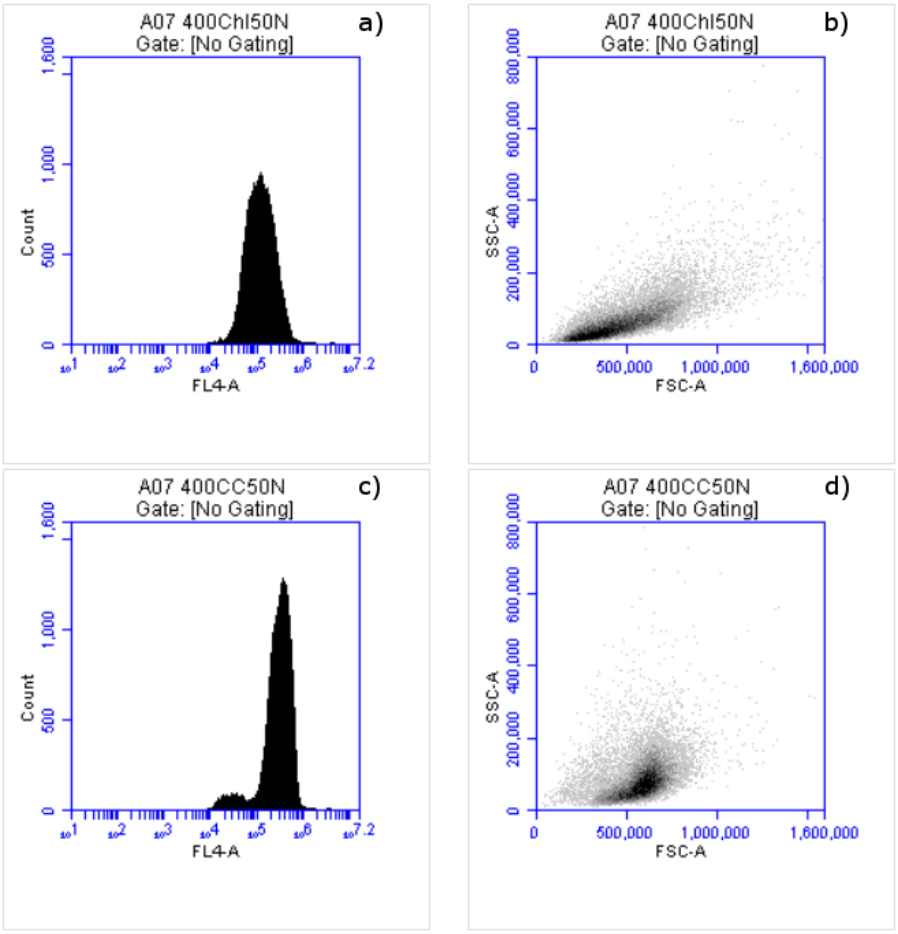


Figure 4.6: Flow cytometry plots of the Chl culture (a and b) and the CC culture (c and d). Plots a and c show a cell count plotted against FL4-A (675±12.5 nm), representing cell size. Plots b and d show side scatter, representing cell complexity, versus forward scatter, representing cell size. Note the presence of the two populations in a and c for the CC culture, with the *Chlorella vulgaris* population in the CC culture having a larger average fluorescence than the *Chlorella vulgaris* population in the Chl culture. Plots b and d show the larger average cell size of the *Chlorella vulgaris* cells in the CC culture compared to those in the Chl culture.

4.1.5 Lipid Content as Percentage of Dry Biomass

Finally, Folch extraction of lipids was performed in triplicate for each of the eight cultures at two different time periods, once when 3 L of each culture was harvested for RNA in the late exponential phase (t=108.5 h for the CC cultures and t=63 h for the Chl cultures) and once when the cultures had reached the stationary phase (t=159.5 h for the CC cultures and t=114 h for the Chl cultures). The lipid content of the cells as percent of dry biomass is presented for the CC cultures in Table 4.5 and the Chl cultures in Table 4.6.

Table 4.5: Folch Extraction Lipid Content (% of dry biomass) for the CC Cultures

Culture Condition	Lipid Content at t=108.5 h	Lipid Content at t=159.5 h
180CC50N	11±1	10±1
180CC100N	13±2	11±1
400CC50N	11±1	8.1±0.2
400CC100N	9.2±0.7	8.8±1.0

Values expressed as mean ± S.D. All analyses were performed in triplicate. Statistical analysis performed using ANOVA with Tukey post-hoc adjustment found p-values for the following effects: irradiance (p=0.0006), nitrate (p=0.3097), time (p=0.0175).

Although the lipid contents for most of the culture conditions show a higher mean lipid content per dry mass in the late exponential phase than in the stationary phase for most of the culture conditions, the effect is only significant at the 95% confidence level for the co-culture samples (p=0.0175). Nonetheless, the lipid contents for all of the cultures at either time are within the range of 8 to 14 percent, and thus the main determining factor of net lipid content in the cultures is dry biomass. A more robust extraction technique, such as Soxhlet extraction, would likely extract more of the available lipids.

Table 4.6: Folch Extraction Lipid Content (% of dry biomass) for the Chl Cultures

Culture Condition	Lipid Content at t=63 h	Lipid Content at t=114 h
180Chl50N	11±1	9.6±0.8
180Chl100N	13±2	13±1
400Chl50N	13±2	14±1
400Chl100N	14±1	11±1

Values expressed as mean ± S.D. All analyses were performed in triplicate. Statistical analysis performed using ANOVA and Tukey post-hoc adjustment found p-values for the following effects: irradiance (p=0.0980), nitrate (p=0.3593), time (p=0.2015).

4.2 Extraction of RNA

4.2.1 Quality Analysis of Total RNA Samples Using UV Spectrophotometry

The A_{260}/A_{280} and A_{260}/A_{230} ratios are provided in Table 4.7. An ideal ratio for A_{260}/A_{280} in approximately 2.0, and an ideal range for the A_{260}/A_{230} ratio is 2.0-2.4 (Farrell 2009).

Table 4.7: A_{260}/A_{280} and A_{260}/A_{230} Absorbance Ratios of Total RNA Samples

Culture Condition	A_{260}/A_{280}	A_{260}/A_{230}
180CC50N	2.13	2.38
180CC100N	2.16	1.06
400CC50N	2.15	2.63
400CC100N	2.16	2.15
180Chl50N	2.19	1.19
180Chl100N	2.15	2.38
400Chl50N	2.16	2.18
400Chl100N	2.14	2.24

All of the samples are close to these criteria for the A_{260}/A_{280}, indicating pure RNA samples relatively free of protein contamination. Five of the eight samples have a A_{260}/A_{230} ratio within the desired range. Nonetheless, while these ratios provide a preliminary indicator of the quality of an RNA sample, the successful use of the sample in downstream applications such as reverse transcription is a better indicator of sample quality.

4.2.2 Gel Electrophoresis of Total RNA Samples

Following extraction of total RNA from frozen samples of each of the eight cultures, DNase treatment, and reverse transcription of each sample, gel electrophoresis was performed.

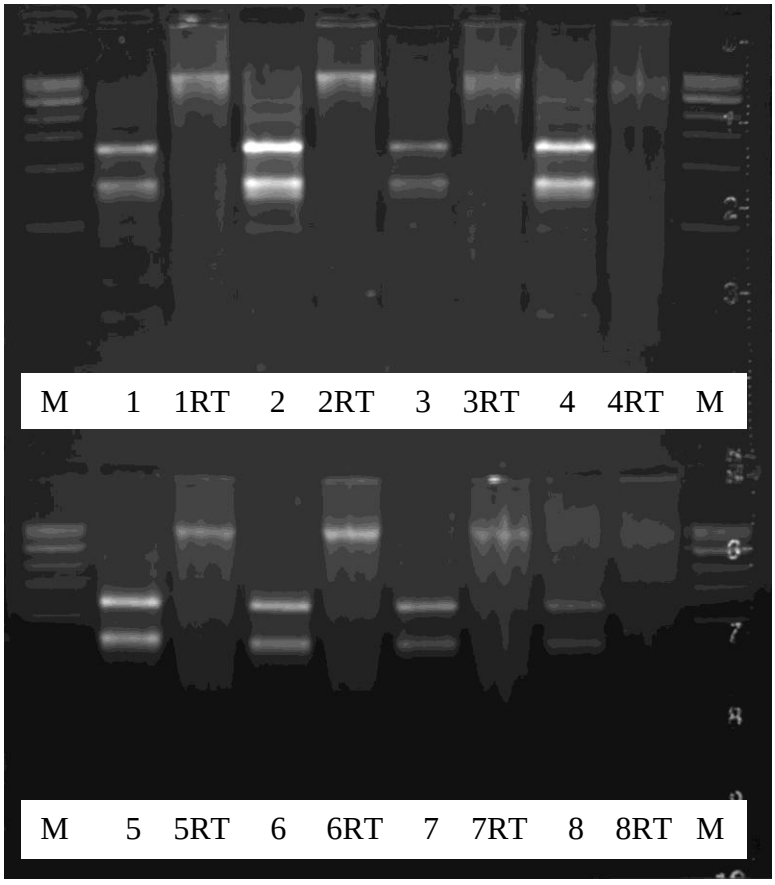


Figure 4.7: Gel of total RNA and reverse transcribed total RNA samples. M refers to the marker, a 1 kb DNA ladder. Numbers refer to sample numbers, with RT referring to the reverse transcribed products. Each total RNA sample shows two strong bands, representing the 28S and 18S ribosomal RNA (rRNA), in addition to mRNA bands. Lanes containing the reverse transcription products show longer bands, indicating successful reverse transcription.

A 1.2% agarose/EtBr gel was loaded with each total RNA sample before and after reverse transcription.

A 1 kb DNA ladder (New England Biosystems, Ipswitch, Massachusetts) was used as a marker. The gel (Figure 4.7) was run at 80 V for 1 hour and transilluminated for 500 ms. The gel shows three important aspects. First, long products in the reverse transcribed sample lanes show that reverse transcription successfully occurred. Second, minimal smearing below the ribosomal RNA (rRNA) bands indicates intact total RNA samples. Finally, minimal fluorescence in the wells for the total RNA samples shows little to no DNA contamination. Thus, the samples were of sufficient quality for downstream applications.

4.3 cDNA Synthesis and Suppression Subtractive Hybridization

Complementary DNA was synthesized and amplified using the SMARTer PCR cDNA Synthesis Kit, and suppression subtractive hybridization was performed using the PCR-Select Subtraction Kit (Clontech, Mountain View, California). Gel electrophoresis was performed several times during the procedures to guarantee successful completion of each step of the protocol. Representative gels are shown below for RsaI digestion, primary PCR, and secondary PCR for four of the eight samples.

Following cDNA synthesis and amplification, the samples were then digested by the restriction endonuclease RsaI, which resulted in the shorter fragments necessary for suppression subtractive hybridization. Gel electrophoresis was performed to determine whether digestion had occurred properly. A 1.2% agarose/EtBr gel was loaded with each cDNA sample before and after RsaI digestion. A GeneRuler™ 1 kb Plus DNA ladder (Fermentas, Glen Burnie, Maryland) was used as a marker. The gel (Figure 4.8) was run at 105 V for 55 min and transilluminated for 1 second. Successful digestion is indicated by the migration of the digested cDNA further down the gel than the undigested cDNA, due to shorter fragments generated due to digestion.

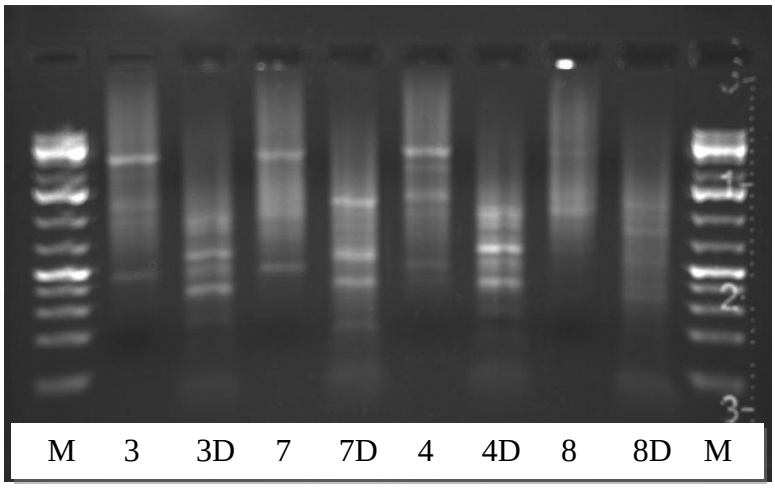


Figure 4.8: Gel of cDNA samples before and after RsaI digestion. M refers to the marker, a 1 kb DNA ladder. Numbers refer to sample numbers, with D referring to the digested products. Bright bands on the DNA ladder correspond to 500 bp, 1500 bp, and 5000 bp. The digested samples migrated farther down the gel, indicating that they are shorter in length due to successful digestion.

Gel electrophoresis was also performed using the primary and secondary PCR products to guarantee that amplification had occurred. A 2% agarose/EtBr gel was loaded with each the primary PCR products of the subtracted cDNA and the corresponding unsubtracted tester control. A GeneRuler™ 1 kb Plus DNA ladder (Fermentas, Glen Burnie, Maryland) was used as a marker. The gel (Figure 4.9) was run at 80 V for 1 hour and transilluminated for 300 ms. A different banding pattern is visible for the unsubtracted tester control, indicating that subtraction was successful. Results match the expected results for the SSH procedure as outlined in the manufacturer's protocol, namely smears in the lanes with some visible bands.

Finally, a 2% agarose/EtBr gel was loaded with each the secondary PCR products of the subtracted cDNA and the corresponding unsubtracted tester control. A GeneRuler™ 1 kb Plus DNA ladder (Fermentas, Glen Burnie, Maryland) was used as a marker.

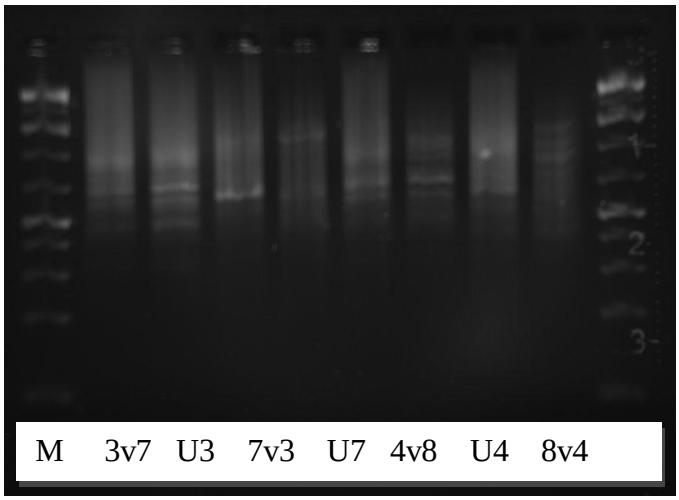


Figure 4.9: Gel of primary PCR products. M refers to the marker, a 1 kb DNA ladder. Numbers correspond to the subtracted library, e.g., 3v7 represents the up3vs7 subtracted library (see Table 3.5). Lanes with the label U correspond to the unsubtracted tester controls. Bright bands on the DNA ladder correspond to 500 bp, 1500 bp, and 5000 bp.

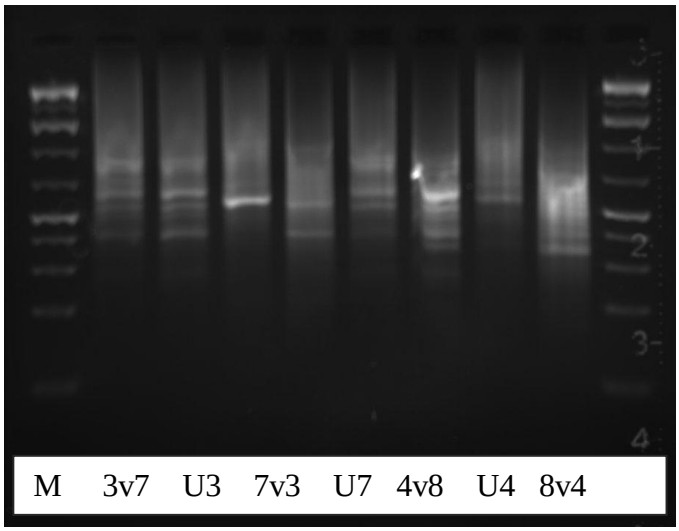


Figure 4.10: Gel of secondary PCR products. M refers to the marker, a 1 kb DNA ladder. Numbers correspond to the subtracted library samples (see Table 3.5), and lanes with the label U correspond to the unsubtracted tester control. Bright bands on the DNA ladder correspond to 500 bp, 1500 bp, and 5000 bp. Different banding patterns between the subtracted library samples and corresponding unsubtracted tester control indicate successful subtractive hybridization.

The gel (Figure 4.10) was run at 80 V for 1 hour and transilluminated for 300 ms. Note that the majority of the PCR products have a length between 500 bp and 1500 bp, the expected length for suppression subtracted hybridization. Banding patterns are different between the subtracted library and the unsubtracted tester control, indicating that the subtractive hybridization process has been successful.

4.4 Sequenced cDNA from Subtracted Libraries

A total of 96 expressed sequence tags (ESTs) were sequenced for each of the 8 subtracted libraries. A total of 694 sequences were obtained after sequences containing no good quality bases or only the cloning vector were removed. The sequences were submitted to the NCBI Expressed Sequence Tags Database (dbEST) database with the ascession numbers JK815763-JK816456. The ESTs were compared to genes in the GenBank database using the BLASTx algorithm (Johnson et al. 2008) through the Blast2Go program (Gotz et al. 2008). The BLASTx algorithm translates the provided nucleotide sequence into a protein in each of the six possible codon reading frames. The resulting protein is compared to proteins in the nr database (a database of known protein sequences for a variety of organisms) at a threshold value of less than or equal to $1x10^{-6}$, as done in a pathway study on the green algae *Dunaliella tertiolecta* (Rismani-Yazdi et al. 2011). Homologous genes were grouped into functional categories, based on categories developed for the model plant *Arabidopsis* (Bevan et al. 1998). A summary of the homologous genes is provided below; a complete list of the homologous genes is provided in Appendix B.

The genes identified by SSH are ones believed to be upregulated in the CC compared to the Chl or the Chl compared to the CC at each growth condition of irradiance and nitrate level. These genes provide key information regarding cell processes for these cultures and give important clues to the effects of irradiance and nitrate level as well as to the effects of co-

culturing the *Chlorella vulgaris* with the *Leptolyngbya* sp. as opposed to growing the *Chlorella vulgaris* without the *Leptolyngbya* sp. Although knowledge of algae genes is limited, the ability to compare the sequences of these upregulated genes to those of other organisms in the databases is very powerful. These comparisons draw on the collected knowledge of genes in not only other algae but also higher plants and other organisms, particularly those photosynthetic organisms for which extensive genetic information is known, such as *Zea mays* (corn).

Table 4.8: Putatively Upregulated Genes Summary

	180Chl50N	180CC50N	180Chl100N	180CC100N	400Chl50N	400CC50N	400Chl100N	400CC100N
Metabolism	4	1	3	9	8	5	3	5
Energy	8	5	4	4	3	2	5	7
Protein Synthesis	7	8	5	5	5	7	7	12
Protein Destination and Storage	1	1	0	5	1	1	1	0
Intracellular Trafficking	1	0	0	3	1	2	0	0
Signal Transduction	1	0	0	3	0	1	0	0
Disease	0	1	0	0	0	0	0	0
Transposons	1	0	0	0	1	0	1	0
Other	0	0	0	1	0	0	0	0

Numbers in the table represent the number of genes in each category. A complete list of the genes is provided in Appendix B.

The number of gene in each group putatively upregulated for each of the eight culture groups is provided in Table 4.8. The genes are referred to as putatively upregulated as further screening for each would be necessary to guarantee upregulation and identify possible false positives, which are known to occur for SSH in some cases (Clontech Laboratories 2011a). Screening techniques that can be used range from lower sensitivity techniques such as dot blot analysis and Northern analysis to higher sensitivity techniques, such as end-point PCR and real-time PCR (Farrell 2009). The higher sensitivity techniques possess the additional advantage that they yield quantitative data regarding the upregulation of the differentially expressed genes identified by SSH.

Many of the genes listed as unknown showed similarity to genes in the database for *Chlorella* sp. or similar green algae, particularly *Chlamydomonas reinhardtii*, *Volvox carteri*, or *Ostreococcus taurii*, but the gene function remains unknown. In some cases, the gene function is also unknown in higher plants for which considerably more gene information exists, such as *Zea mays* (corn), *Arabidopsis thaliana* (thale cress, the most commonly used model plant in genomics work), *Medicago truncatula* (a model plant used to study interaction with nitrogen-fixing bacteria), *Oryza sativa* (rice), and *Glycine max* (soybean).

Also illustrative is a comparison of upregulation by Chl versus CC culture, 50% nitrate level versus 100% nitrate level, and 180 µmol/m^2-sec versus 400 µmol/m^2-sec irradiance (Table 4.9). The majority of the genes that had matches in the databases fall into the metabolism, energy, and protein synthesis categories. The most significant contributions to the entire pool of sequences are energy-related genes from the subtracted libraries of the Chl cultures, the cultures grown starting at the 100% nitrate level, and the cultures grown at the 400 µmol/m^2-sec irradiance level.

A number of interesting genes are present in the libraries. First, a gene for auxin binding protein 1 appears in one of the libraries of genes putatively upregulated in a Chl culture. If the gene is indeed upregulated in *Chlorella vulgris* in the Chl compared to *Chlorella vulgaris* in the CC, it would suggest that some sort of non-photosynthetic bacteria in the Chl culture are producing auxins. Second, two genes related to the production of lipids, major oil drop protein and acyl-CoA binding protein, appear, in addition to a gene related to the production of carotenoids, 15-cis-zeta-carotene isomerase. Finally, there are a large number of putatively differentially expressed genes corresponding to the light reactions of photosynthesis in libraries

for both the Chl and CC cultures. For example, four of the key subunits of Photosystem II, cytochrome b_{559}, D1, D2, and CP47, are present in at least one subtracted library.

Table 4.9: Culture Condition Summary of Genes in Subtracted Libraries

	Chlorella upregulated	Co-culture upregulated	50% NO_3	100% NO_3	180 irradiance	400 irradiance
metabolism	28	19	22	25	22	25
energy	134	52	79	107	83	103
protein synthesis	57	55	57	55	51	61
protein destination and storage	3	10	4	9	10	3
intracellular trafficking	2	5	4	3	4	3
signal transduction	1	4	2	3	4	1
disease	0	1	1	0	1	0
transposons	5	2	6	1	3	4
other	0	1	0	1	1	0
unknown	120	202	171	151	179	143
TOTAL	350	351	346	355	358	343

Numbers in the table represent the number of sequenced clones corresponding to genes in each category. A complete list of the genes is provided in Appendix B.

4.5 Quantitative PCR

Quantitative PCR (qPCR) was performed on nine genes. A list of the genes chosen for qPCR, the functional classification of the gene (Bevan et al. 1998), and the reason each was chosen for qPCR analysis is provided in Table 4.10.

The first four genes in Table 4.10 represent genes for which expressed sequence tags were found in the subtracted libraries corresponding to genes upregulated in *Chlorella* sp. or in the co-culture grown at three or more of the four culture conditions. The fifth gene, ATP synthase subunit alpha, is essential to energy production in the cell, as ATP along with NADPH provide energy for photosynthetic carbon fixation. The remaining genes were chosen to span categories of interest, particularly stress response, signaling, and lipid production. Since green

algae are known to synthesize lipids under stressed conditions, such as nitrate depletion, stress response genes are important to examine.

Table 4.10: Genes Selected for Expression Analysis Using Quantitative PCR

Gene	Functional Category	Comments
Photosystem II apoprotein CP47	Energy	In all four SSH libraries of genes upregulated in monoculture *Chlorella*; one of the key proteins in Photosystem II
60S ribosomal protein L23a	Protein Synthesis	In all four SSH libraries of genes upregulated in co-culture *Chlorella*
Cytochrome b559 alpha subunit	Energy	In three SSH libraries of genes upregulated in monoculture *Chlorella*; one of the key proteins in Photosystem II
Photosystem I Reaction Center XI	Energy	In all four SSH libraries of genes upregulated in co-culture *Chlorella*; one of the key proteins in Photosystem I
ATP synthase subunit alpha	Metabolism	Important to energy production in the cell
Molecular chaperone (HSP family)	Protein Destination and Storage/Stress-Related	In a family of heat-shock proteins, related to stress response
Light-harvesting chlorophyll-a/b binding	Energy	Found upregulated significantly in stressed conditions (Machida et al. 2008; Rodolfi et al. 2009); regulates the ability of the cell to use light energ
Phosphoprotein phosphatase	Metabolism/Stress-Related	Essential to cell signaling processes and cytoskeletal structure; inhibition of these phosphatases can be detrimental to an organism (Toivola and Eriksson 1999)
Oil globule associated protein	Energy	Analogous proteins found in *Dunaliella* and *Haematococcus*; important to lipid storage

Cell signaling is also important to examine, particularly with respect to stress response. For example, chlorophyll-a/b binding protein was found to be upregulated over 400 times relative to actin, a reference gene, in a study done on freeze-hardening in *Chlorella vulgaris*

(Machida et al. 2008). Finally, the oil globule associated protein is of particular interest due to its role in lipid storage in microalgae. Oil globule associated proteins have been found in *Dunaliella salina* (Davidi et al. 2012) and *Haematococcus pluvialis (*Peled et al. 2011), other green algae. As of this writing, detailed work characterizing the corresponding protein in *Chlorella vulgaris* has not yet been published.

For each of these nine genes, the qPCR results for the eight samples, in addition to a brief literature review of the gene will be provided. Several of the genes are in photosynthetic pathways, so an understanding of their position and function in the entire photosynthetic scheme is important. The current state of knowledge about the structure and pathways of photosynthesis in the green algae *Chlamydomonas reinhardtii* as well as the antenna complexes are presented in Figures 4.11 and 4.12, respectively (Laboratories 2010; Laboratories 2012).

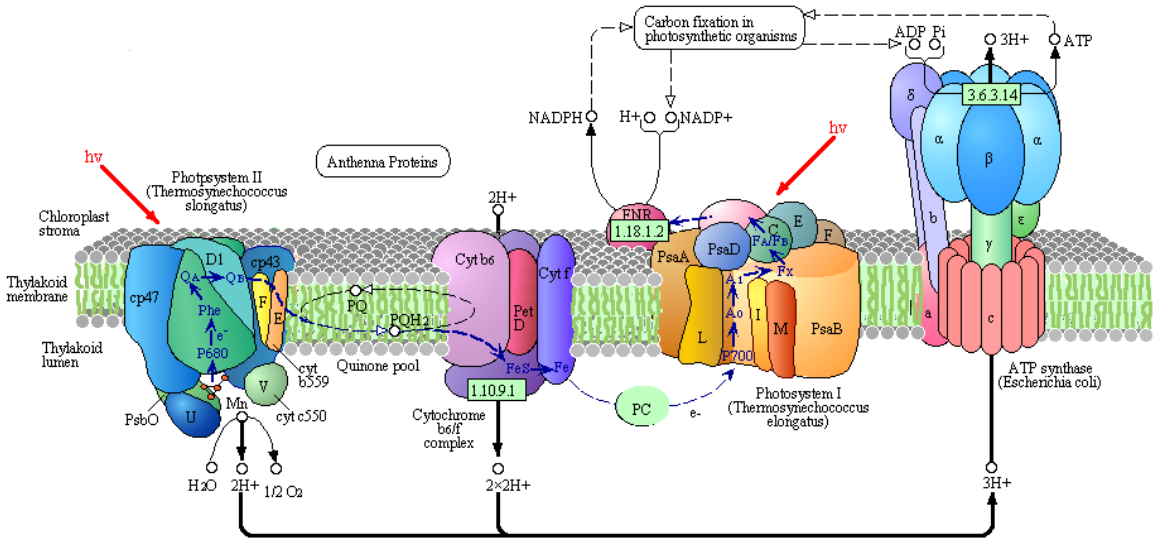


Figure 4.11: Photosynthesis proteins and pathways in *Chlaymdomonas reinhardtii* (adapted from Kanehisa Laboratories 2012).

Since *Chlorella vulgaris* is closely related and many of these genes are known to be well-conserved, it is expected that *Chlorella vulgaris* will contain homologous genes.

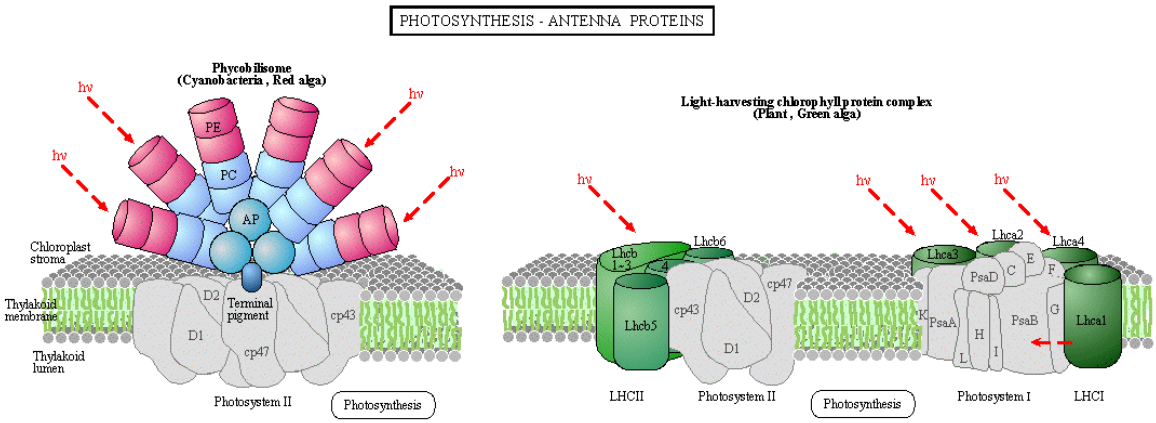


Figure 4.12: Light-harvesting chlorophyll protein complex in *Chlamydomonas reinhardtii* (adapted from Kanehisa Laboratories 2010). On the left is shown the analogous antenna complex in cyanobacteria and red algae, which includes phycocyanin and phycoerythrin.

4.5.1 Photosystem II Apoprotein CP47

Expressed sequence tags containing sequences homologous to Photosystem II apoprotein CP47 (psbB) were found in the subtracted libraries for genes upregulated in *Chlorella vulgaris* for all four culture conditions. Gene expression results for PSII apoprotein CP47 are presented in Figure 4.13. In all four culture conditions, expression of CP47 in the Chl culture exceeded that of expression in the CC at the 99.9% confidence level. For the 4 Chl cultures, the effect of irradiance ($p=0.0931$) was not significant, although the effect of nitrate level was ($p=0.0152$). For the 4 CC cultures, the effect of irradiance ($p=0.0130$) was significant, although the effect of nitrate level was not ($p=0.7316$). Nonetheless, the effects of irradiance ($p=0.8309$) and nitrate level ($p=0.3259$) were not statistically significant across the 8 cultures.

CP47 is one of approximately 20 subunits in the Photosystem II (PSII) complex. CP47 coordinates 16 chlorophylls and 2-3 β-carotenes each, transmitting excitation energy from the antenna proteins to the reaction center, which is formed by D1 and D2 (Spyridaki et al. 2006;

Wydrzynski 2008; Reppert et al. 2010; Croce and van Amerongen 2011). Approximately 80% of the protein is conserved among photosynthetic organisms (Spyridaki et al. 2006).

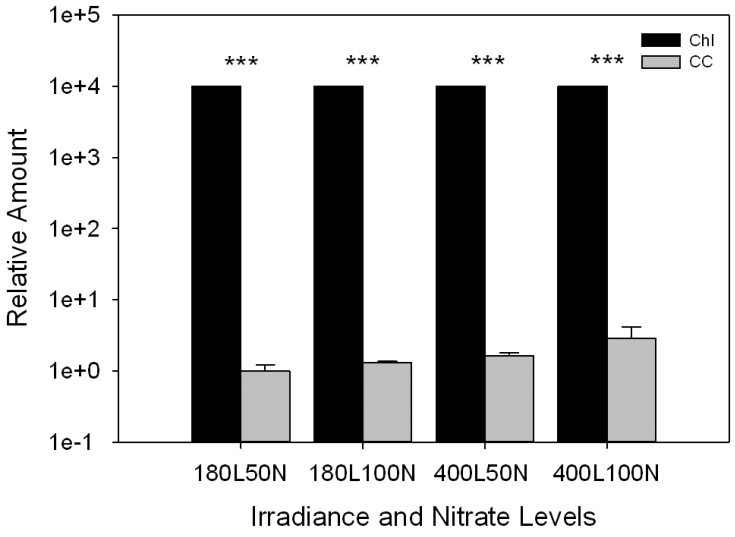


Figure 4.13: Gene expression of PSII apoprotein CP47 normalized to the lowest expression level among the 8 cultures. Values are mean ± S.E. from triplicates. Statistical significance of expression level difference between Chl and CC cultures at the same irradiance and nitrate level indicated by *** ($p<0.001$). P-values for effects of irradiance and nitrate on gene expression levels are 0.8309 and 0.3259, respectively. Mean values above 10000 are reported as 10000.

The protein is known to be present in a trimer of dimers in each reaction center (Eaton-Rye and Putnam-Evans 2005), and it is involved in the sequestration of Cl^- for the oxygen evolving complex (OEC), the set of proteins that splits water in PSII (Bricker and Frankel 2002). Although CP47 is more thermally stable than the corresponding protein CP43, it is more susceptible to damage by light (Wang et al. 1999).

The gene regulation of CP47 in reference to other proteins in the PSII complex has been studied in depth. In *Chlamydomonas* the sequence of assembly of the reaction center of PSII proceeds by control by epistasy of synthesis (CES), "whereby the presence of one subunit is required for sustained synthesis of another chloroplast-encoded subunit from the same protein

complex" (Minai 2006). Synthesis begins with the accumulation of cytochrome b_{559}, followed by synthesis of D2, D1, subunits PsbI and PsbW, and finally CP43 and CP47. Control on the synthesis of D1 and CP47 occurs at the step of translation initiation, with D2 controlling translation of D2 and D1 controlling transaltion of CP47. Thus mRNA transcripts encoding for D1 and CP47 can accumulate, but translation into the proteins will not occur. This process is summarized in Figure 4.14.

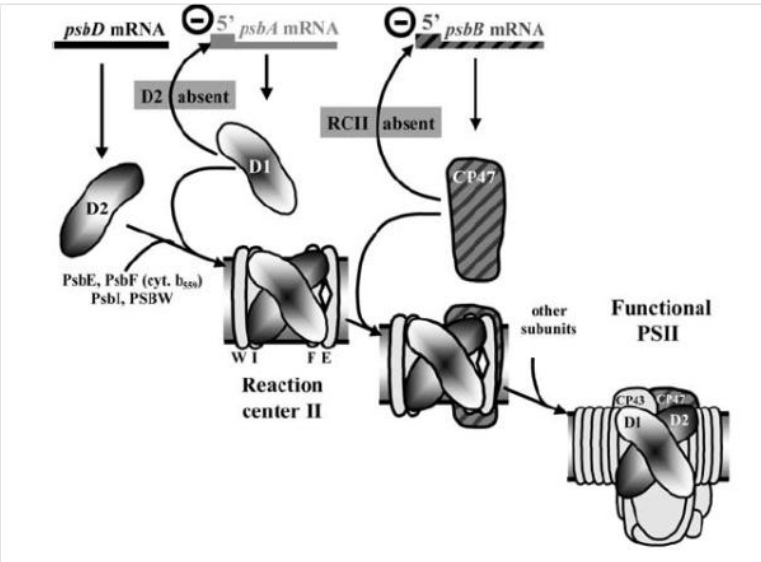


Figure 4.14: Translational control in the assembly of the PSII reaction center complex (Minai 2006).

Nonetheless, the CES assembly process does not occur during photoinhibition but rather only during *de novo* synthesis of the PSII complex (Minai 2006). Recovery from photoinhibition, which damages the D1 protein, involves the partial de-assembly of the PS II complex, rapid synthesis of new D1 protein and insertion into the complex, and reactivation of the complex (Melis 1999). Thus, the abundance of mRNA transcripts for CP47 in the Chl cultures samples does not imply that photodamage has occurred, resulting in an inability to translate CP47 mRNA into protein due to the lack of D1 in the reaction centers. Rather, CP47

transcript abundance could reflect that in the synthesis of new PSII complexes, D1 has not yet been assembled. Furthermore, photoinhibition would be more pronounced at the higher irradiance level, whereas CP47 mRNA levels are higher for the Chl culture at the lower irradiance level.

To better understand and compare *de novo* synthesis and photodamage repair of Photosystem II, mRNA levels of D2, D1 and CP47 would likely all need to be measured. Additionally, comparison to transcript levels during conditions that induce photodamage and in the time period corresponding to repair would yield more information regarding assembly and repair of new and damaged PSII complexes, respectively, in both the Chl and CC cultures.

4.5.2 60S ribosomal protein L23a

Expressed sequence tags containing sequences homologous to 60S ribosomal protein L23a were found in the subtracted libraries for genes upregulated in the co-culture for all four culture conditions. Gene expression results for 60S ribosomal protein L23a are presented in Figure 4.15. Expression of mRNA for L23a was upregulated in the CC cultures compared to the Chl cultures at the 99% or higher confidence level. Across the 4 Chl and across the 4 CC cultures, the effect of irradiance was not significant ($p=0.3680$ and $p=0.5887$, respectively). Nonetheless, among the 4 Chl and the 4 CC cultures, the effect of nitrate level was significant ($p=0.0303$ and $p=0.0087$, respectively). Across the 8 cultures, the effects of irradiance ($p=0.9202$) and nitrate level ($p=0.8994$) were not statistically significant.

Ribosomal protein L23a is a member of the L23/L25 family found in all organisms (Degenhardt and Bonham-Smith 2008b). Two paralogs of the protein have been found in *Arabidopsis*, in which L23a is one of 81 ribosomal proteins. Elimination of the B form of the protein does not affect growth, but elimination of the A form results in growth retardation and abnormal leaf and root morophology (Degenhardt and Bonham-Smith 2008a).

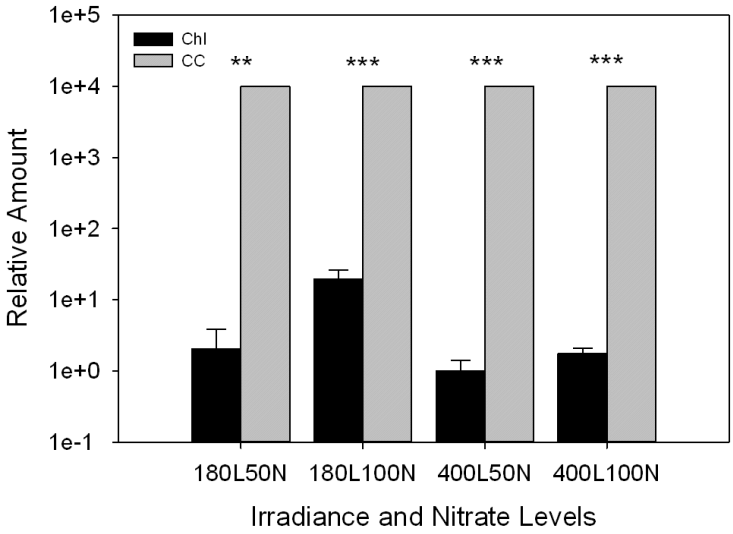


Figure 4.15: Gene expression of 60S ribosomal protein L23a normalized to the lowest expression level among the 8 cultures. Values are mean ± S.E. from triplicates. Statistical significance of expression level difference between Chl and CC cultures at the same irradiance and nitrate level indicated by ** ($p<0.01$) and *** ($p<0.001$). P-values for effects of irradiance and nitrate on gene expression levels are 0.9202 and 0.8994, respectively. Mean values above 10000 are reported as 10000.

Since L23a is located on the ribosome complex near where the polypeptide being assembled exits the complex, it is believed to be involved in protein translation and secretion (Degenhardt and Bonham-Smith 2008a).

Transcript levels of L23a in *Arabidopsis* were measured in response to temperature stress, wounding, copper sulfate stress, and application of the phytohormones indole-3-acetic acid (IAA), 6-benzylaminopurine (a cytokinin), abscisic acid (ABA), and the gibberellins GA_3 (McIntosh and Bonham-Smith 2005). Both forms of L23a were upregulated compared to a control in the IAA and 6-benzylaminopurine treatments, and one form was downregulated in the copper sulfate stress treatment sample. Expression of L23a following temperature stress and wounding remained stable compared to the control. Although cytokinin genes and only one

auxin gene were identified in the subtracted libraries, *Chlorella* sp. is known to be affected by the phytohormones IAA and cytokinins. Upregulation of L23a could indicate the possible presence of auxins or cytokinins in the culture media, a factor that should be tested through an assay for these phytohormones. Finally, both paralogs of the gene showed upregulation 1-5 hours following photoinhibition, followed by a drop in transcript levels; one explanation given is that response to oxidative stress caused by photoinhibition might require increased translational capacity, resulting in higher levels of mRNA expression this ribosomal protein (Degenhardt and Bonham-Smith 2008b).

The role of L23a in signaling has been examined with respect to signal recognition particles (SRP), which are present in all organisms (Pool 2005). The 54 kDa signal recognition particle SRP54 binds to ribosome in contact with ribosomal protein L23a, and it is involved in recognition of the N-terminal hydrophobic portion of polypeptides destined for transport to the membrane. If the signal peptide is found, the SRP54 mediates the transport of the protein to the membrane. Contact with L23a changes the conformation of SRP54 to increase its affinity for both binding the signal peptide and binding GTP, which stabilizes the complex formed by the SRP, the ribosome, and the nascent polypeptide. Several expressed sequence tags encoding GTP-binding proteins were found in the subtracted libraries.

A chloroplast homolog to SRP54, cpSRP54, has been found in the chloroplast of *Chlorella vulgaris* and other algae but not in higher plants. One function of cpSRP54 is the formation of a complex with another SRP, cpSRC43, and light-harvesting chlorophyll a/b binding protein, in which the SRP functions to insert the light-harvesting protein into the membrane. This complex of three proteins comprises nearly one-third of the total protein

content of the thylakoid membrane. It is interesting to note that mitochondria use a different pathway than the SRP for insertion of proteins into their membrane.

Although the role of L23a is not completely understood even in higher plants, its involvement in the identification of proteins for insertion into a membrane or secretion is important in light of the co-culture. The role of small excreted polypeptides in the association between model plant *Medicago truncatula* and nitrogen-fixing soil bacteria has been identified, with the polypeptides possibly involved in cell-to-cell signaling or defense (Mergaert 2003). Identification of similar genes in the *Chlorella variabilis* genome, their expression in the co-culture, and the mediation secretion through L23a and SRP would provide a explanation for enhanced characteristics of the CC versus the Chl culture and provide insight into whether the cyanobacteria is fixing nitrogen.

4.5.3 Cytochrome b559 Alpha Subunit

Expressed sequence tags containing sequences for the alpha subunit of cytochrome b_{559} were found in the subtracted libraries for genes upregulated in the Chl cultures for three of the four culture conditions. mRNA transcripts for the subunit were found to be upregulated in the Chl cultures compared to the CC cultures for all 4 culture conditions at the 99.9% confidence level (Figure 4.16). For the 4 Chl cultures, neither the effects of irradiance ($p=0.3290$) nor nitrate level ($p=0.4199$) on expression level were significant. For the 4 CC cultures, neither was significant as well (irradiance, $p=0.7771$; nitrate level, $p=0.1905$). Finally, the effects of irradiance ($p=0.6397$) and nitrate level ($p=0.4343$) were not statistically significant across the 8 cultures.

As previously discussed, cytochrome b559 is the first component to assemble in the biogenesis of new Photosystem II complexes (Minai 2006). Abundance of b_{559} transcripts in the cells, especially in cases of light-limited growth, could be the result of the cell producing more

PSII complexes for additional light harvesting. Nonetheless, gene expression levels are not significantly higher for the lower irradiance cultures, as would be expected if the cells were expressing cytochrome b_{559} to assemble more PSII complexes.

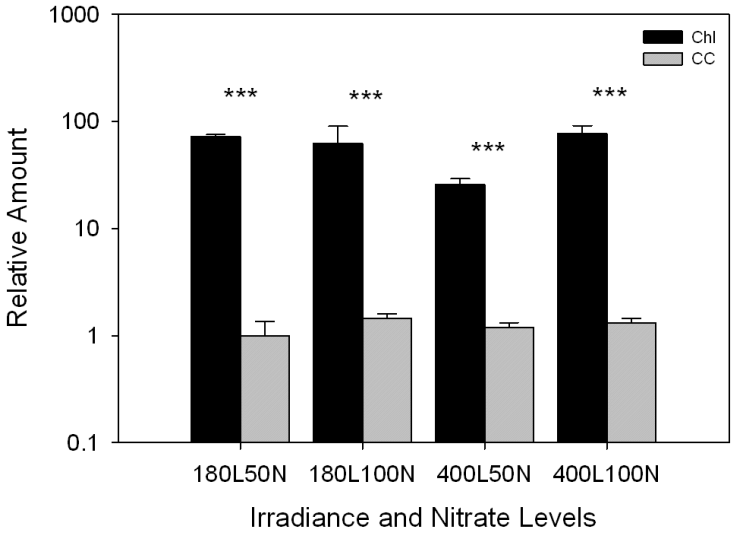


Figure 4.16: Gene expression of cytochrome b559 alpha subunit normalized to the lowest expression level among the 8 cultures. Values are mean ± S.E. from triplicates. Statistical significance of expression level difference between Chl and CC cultures at the same irradiance and nitrate level indicated by *** ($p<0.001$). P-values for effects of irradiance and nitrate on gene expression levels are 0.6397 and 0.4343, respectively.

Despite many studies on the topic, the function of cytochrome b_{559} remains unknown. Nonetheless, it is known not to participate in the primary electron transfer reactions leading to the splitting of water in PS II (Stewart and Brudvig 1998; Kropacheva et al. 2003; Shinopoulos and Brudvig 2012). Furthermore, cytochrome b_{559} is the first subunit of PSII to assemble and must be present before D2 and D1 are assembled in the reaction center (Minai 2006; Spyridaki et al. 2006). It is widely believed to play a role in protection against light stress, as indicated by increased photoinhibition in strains of algae in which the gene that encodes b_{559} has been

mutated (Shinopoulos and Brudvig 2012). The cytochrome is involved a cyclic pathway of secondary electron transfer, in which it donates an electron to $P_{680}^{\cdot+}$ through $Chl^{\cdot+}$ or $Car^{\cdot+}$ and then receives an electron from the reduced plastoquinone (PQ).

This cyclic pathway of electron transfer allows cytochrome b_{559} to limit damage caused by reactive oxidative species (ROS) generated by $P_{680}^{\cdot+}$ in situations in which electron transfer from H_2O to $P_{680}^{\cdot+}$ is impaired, during photinhibition and when the oxygen evolving complex is being assembled (Shinopoulos and Brudvig 2012).

In the case of photoinhibition, it would be expected that cytochrome b_{559} would be more upregulated in the higher irradiance culture condition. Nonetheless, expression levels do not significantly vary due to irradiance level, suggesting that the upregulation of b_{559} in the Chl cultures is not due to photoinhibition.

4.5.4 Photosystem I Reaction Center Subunit XI

Expressed sequence tags containing sequences for Photosystem I reaction center subunit XI were found in the subtracted libraries for genes upregulated in the co-culture for all four culture conditions. Upregulation in the CC cultures was present at all four culture conditions at the 99.9% confidence level (Figure 4.17). For the 4 Chl cultures, the effect of irradiance on expression level was not significant at the 95% confidence level (p=1.0000), although the effect of nitrate was (p=0.0022). For the 4 CC cultures, neither effect was statistically significant (irradiance, p=0.7316; nitrate, p=0.4199). Likewise, across all 8 cultures, neither the effects of irradiance (p=0.8998) not nitrate level (p-0.1829) were significant.

Photosystem I (PSI) catalyzes the reduction $NADP^+$ to NADPH, which along with the production of ATP, provides energy for carbon fixation in photosynthetic organisms. Although PSI has a trimeric form in cyanobacteria, it is known to have a monomeric form in

Chlamydomonas reinhardtii and higher plants (Chitnis and Chitnis 1993; Kargul 2003). PSI is highly conserved among organisms, with 14 subunits shared in higher plants and green algae.

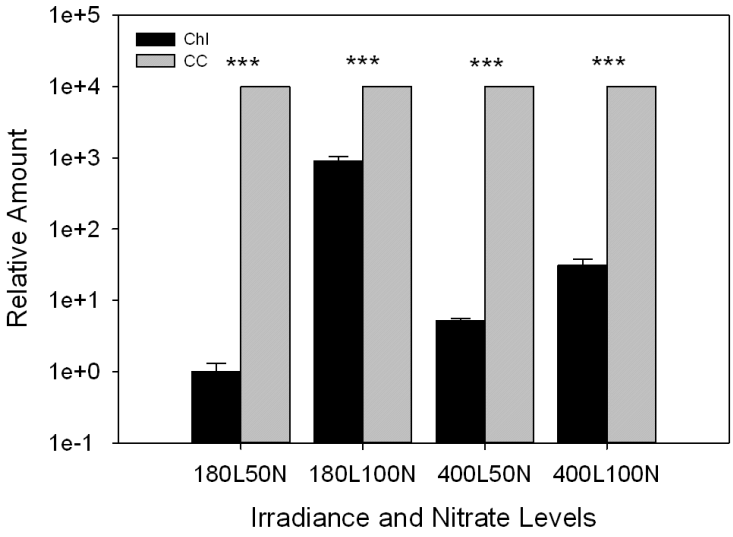


Figure 4.17: Gene expression of Photosystem I reaction center subunit XI normalized to the lowest expression level among the 8 samples. Values are mean ± S.E. from triplicates. Statistical significance of expression level difference between Chl and CC samples at the same irradiance and nitrate level indicated by *** ($p<0.001$). P-values for effects of irradiance and nitrate on gene expression levels are 0.8998 and 0.1829, respectively. Mean values above 10000 are reported as 10000.

The photosystem coordinates approximately 100 chlorophyll and 20 β-carotene molecules (Drop et al. 2011). Nonetheless, the role of PSI reaction center subunit XI (PsaL) remains mysterious. Since green algae and plants do not have a trimeric PSI as cyanobacteria do, PsaL is believed to play a different role. A close association between PsaH and PsaL has been observed, and an *Arabidopsis* mutant in which PsaH was removed resulted in PsaL comprising 50% of the proteins of PS I (Naver et al. 1999).

One possible explanation for upregulation of Photosystem XI reaction center subunit XI may be an attempt by the cell to increase the activity of PSI in the CC cultures. A study of gene expression for other genes in PSI would be beneficial in testing the hypothesis.

4.5.5 ATP Synthase Subunit Alpha

Expressed sequence tags containing sequences for ATP synthase subunit alpha were found in 1 subtracted library for genes upregulated in the co-culture. Expression levels of the gene were upregulated in all four Chl cultures compared to the CC cultures at the 99.9% confidence level (Figure 4.18).

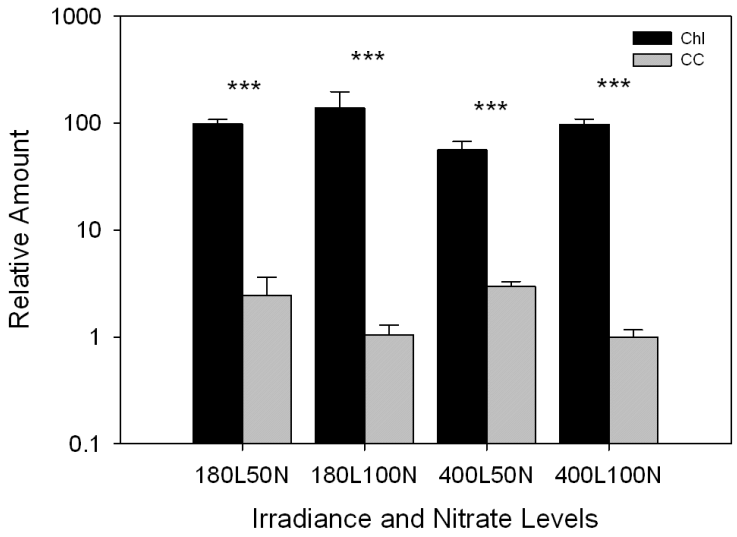


Figure 4.18: Gene expression of ATP synthase subunit alpha normalized to the lowest expression level among the 8 cultures. Values are mean ± S.E. from triplicates. Statistical significance of expression level difference between Chl and CC cultures at the same irradiance and nitrate level indicated by *** ($p<0.001$). P-values for effects of irradiance and nitrate on gene expression levels are 0.8108 and 0.6593, respectively.

For the 4 Chl cultures, neither the effects of irradiance ($p=0.2403$) nor nitrate level ($p=0.3939$) on expression were significant. For the 4 CC cultures, the effect of irradiance ($p=0.6190$) was not, but the effect of nitrate level was ($p=0.0216$). Across the 8 cultures, neither of the effects was significant (irradiance, $p=0.8108$; nitrate level, $p=0.6593$).

ATP synthase is an important biological complex of over 500 kDa that consists of two rotating motors (Okuno et al. 2011). A diagram of the different subunits is provided in Figure 4.9. ATP synthase plays a key role in the production of ATP, which along with the production of NADPH, provides energy for carbon fixation in photosynthetic organisms. Additionally, ATP synthase is present in mitochondria, whereby energy is generated by breaking down carbohydrates.

A deeper understanding of the regulation of ATP synthase subunit alpha would be aided by a comparison to transcript levels for the enzymes resulting in NADPH production, as the balance between the supply of NADPH and ATP is important to the photosynthetic productivity of the organism and is a useful topic for exploration through metabolic engineering (Kramer and Evans 2010).

One additional interesting trait of ATP synthase is that the enzyme is a known target of microcystins, a type of heptapeptide produced by many cyanobacteria and known to inhibit type 1 (PP1) and type 2 (PP2A) protein phosphatases (Mikhailov et al. 2003). The possible role of microcystins in differential gene expression in the Chl and CC cultures will be discussed in further detail later.

4.5.6 Molecular Chaperone (HSP family)

Expressed sequence tags containing sequences for a molecular chaperone in the heat shock protein family was found in 1 subtracted library for genes upregulated in the co-culture. The gene was upregulated for all four CC cultures, with a statistical significance at the 99.9% confidence level (Figure 4.19). For the 4 Chl cultures, the effects of irradiance ($p=0.5887$) was not significant, but the effect of nitrate level ($p=0.0260$) on expression was. On the other hand, neither effect was significant for the CC cultures (irradiance, $p=1.0000$; nitrate level, $p=0.1970$).

Additionally, across the 8 cultures, neither effect was significant (irradiance, p=0.8208; nitrate level, p=0.7670).

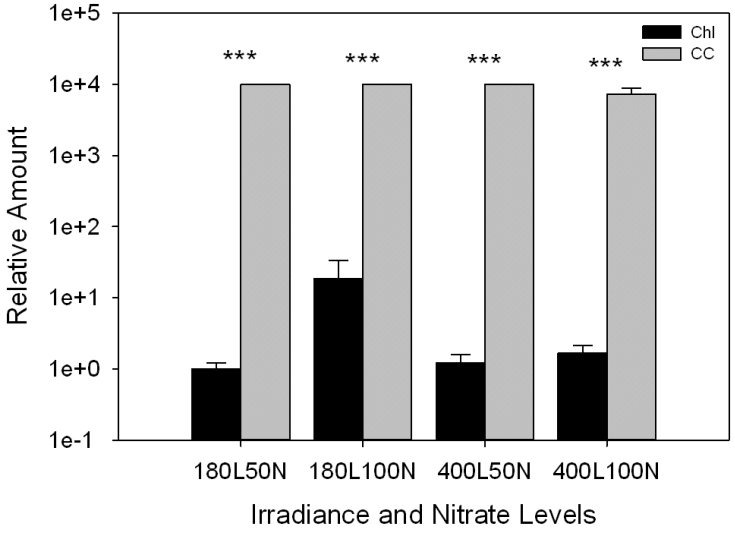


Figure 4.19: Gene expression of molecular chaperone (heat shock protein family) normalized to the lowest expression level among the 8 cultures. Values are mean ± S.E. from triplicates. Statistical significance of expression level difference between Chl and CC cultures at the same irradiance and nitrate level indicated by *** ($p<0.001$). P-values for effects of irradiance and nitrate on gene expression levels are 0.8308 and 0.7670, respectively. Mean values above 10000 are reported as 10000.

The role of heat shock proteins in response to not only heat shock but also salinity stress and photoinhibition has been investigated heavily in algae, including the green macroalga *Ulva fasciata* (Sung et al. 2010) and green microalgae *Dunaliella salina* (Yokthongwattana et al. 2001) and *Chlamydomonas reinhardtii* (Kropat et al. 1997; Schroda et al. 1999). Heat shock proteins are known to stabilize proteins by promoting proper protein folding and maintaining their structural integrity (Sung et al. 2010). High salt stress has been known to generate reactive oxygen species, triggering protein degradation through ubiquitin-proteosome pathway (Sung et al. 2010).

Heat shock proteins have been shown to be induced by transfer of algae from low light to high light conditions (Kropat et al. 1997; Schroda et al. 1999; Yokthongwattana et al. 2001). In particular, green algae lack a pathway for the repair of the D1 subunit of Photosystem II following photoinhibition. While higher plants have a phosphorylation pathway for damage and repair, the isolation of a complex of heat shock protein HSP70B, damaged but undegraded D1, D2, and CP47 in *Dunaliella* suggests that the chloroplast-encoded HSP70B is involved in photosystem repair (Yokthongwattana et al. 2001). Evidence in *Chlamydomonas* suggests that HSP70B may be involved in photoinhibition response either by reactivation of the electron acceptor side of PS II or by limiting the targeting of undamaged D1 for destruction (Schroda et al. 1999). The role of the protein in repair was further supported by significant changes in the ability of the cell to cope with photodamage due to only slight changes in the abundance of HSP70B (Schroda et al. 1999).

The role of heat shock proteins in response to microcystin treatment has also been investigated in *Synechocystis* sp. PCC6803 (Li et al. 2009). In particular, a heat shock protein found upregulated in cultures treated with microcystin MC-RR. In this case, the protein is believed to play dual roles of preventing malfolding of antioxidant proteins synthesized to prevent oxidative damage and protect against stress-induced apoptosis. The possible role of microcystins in differential gene expression in the Chl and CC cultures will be discussed in further detail later.

4.5.7 Light-Harvesting Chlorophyll Binding Protein

Expressed sequence tags containing sequences for light-harvesting chlorophyll binding proteins (LHCB) were found in 2 subtracted libraries for genes upregulated in the CC cultures and 2 subtracted libraries for genes upregulated in the Chl cultures. Interestingly, two of the libraries

corresponded to genes upregulated at the lower irradiance level and two to genes upregulated at the higher irradiance level.

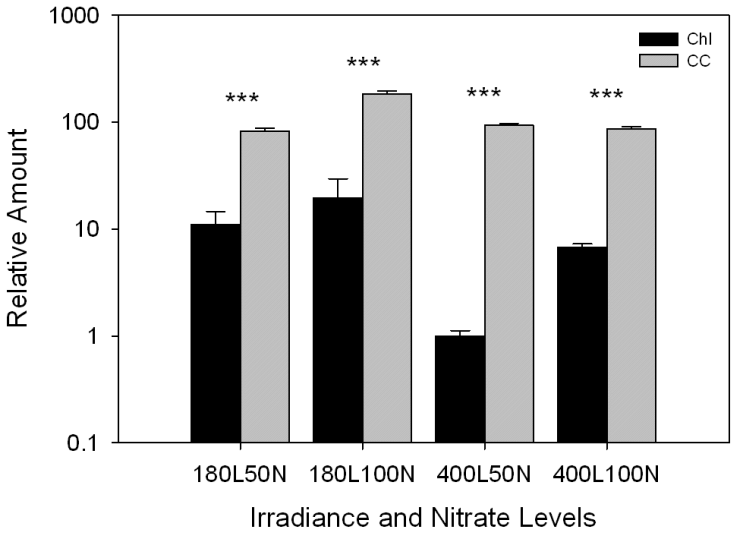


Figure 4.20: Gene expression of light-harvesting chlorophyll binding protein normalized to the lowest expression level among the 8 cultures. Values are mean ± S.E. from triplicates. Statistical significance of expression level difference between Chl and CC cultures at the same irradiance and nitrate level indicated by *** ($p<0.001$). P-values for effects of irradiance and nitrate on gene expression levels are 0.2475 and 0.4182, respectively.

For all four culture conditions, expression levels of LHCB were upregulated in the CC cultures compared to the Chl cultures at the 99.9% confidence level (Figure 4.20). At the 95% confidence level, the effect of nitrate level on gene expression was not statistically significant either for the 4 Chl cultures ($p=0.1926$), but the effect of nitrate level was ($p=0.0173$). For the four CC cultures, neither effect was significant (irradiance, $p=0.3939$; nitrate level, $p=0.3939$). Likewise, neither effect was significant across the eight cultures (irradiance, $p=0.2475$; nitrate level, $p=0.4182$).

Regulation of mRNA transcripts for, and subsequent levels of, light-harvesting chlorophyll binding proteins (LHCBs) is controlled by several factors. In the case of continuous

irradiance, in which the culture for this study were grown, the mRNA abundance is regulated by the redox conditions of photosynthetic electron transport (Surpin and Chory 2002).

A study on *Chlorella vulgaris* grown 5°C and 27°C and irradiance levels of 50 µmol/m^2-sec and 150 µmol/m^2-sec showed that levels of reactive oxygen species correlate strongly with levels of light-harvesting chlorophyll-binding protein at both temperatures, suggesting that dual signaling mechanisms of ROS production in the electron transport chain along with the redox conditions of the plastoquinone pool regulate the production of LHCBs (Wilson et al. 2002).

Additionally, adaptation of the cells to a change from high irradiance to low irradiance, which would be caused by continually increasing cell density in the media due to growth, results in an abundance of mRNA encoding the LHCBs. The translation of the mRNA into LHCBs occurs and is followed by increases in the synthesis of photosystem reaction centers and membranes (Falkowski and Raven 1997). Transcripts for PSI reaction center XI (PsaL) were upregulated in the CC cultures, which would be consistent with the synthesis of reaction centers following synthesis of antenna proteins.

4.5.8 Phosphoprotein Phosphatase

Expressed sequence tags containing sequences for phosphoprotein phosphatase were found in 2 subtracted libraries for genes upregulated in the CC cultures. The gene was upregulated in the CC cultures compared to the Chl cultures at the 99.9% confidence level for all 4 culture conditions (Figure 4.21). For the 4 Chl cultures, neither the effects of irradiance ($p=0.1061$) not nitrate level ($p=0.0606$) on expression level were significant. Nonetheless, for the 4 CC cultures, the effect of irradiance was statistically significant ($p=0.0022$), while the effect of nitrate level

was not (0.8182). Across the 8 cultures, neither effect was significant (irradiance, p=0.1088; nitrate level, p=0.4414).

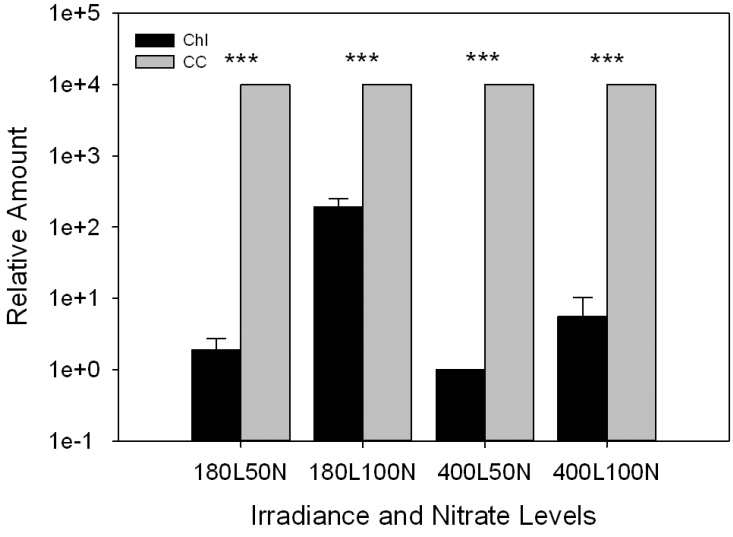


Figure 4.21: Gene expression of phosphoprotein phosphatase normalized to the lowest expression level among the 8 cultures. Values are mean ± S.E. from triplicates. Statistical significance of expression level difference between Chl and CC cultures at the same irradiance and nitrate level indicated by *** ($p<0.001$). P-values for effects of irradiance and nitrate on gene expression levels are 0.1088 and 0.4714, respectively. Mean values above 10000 are reported as 10000.

Phosphoprotein phosphatases (PPPs) are one of three classes of enzymes that remove a phosphate group from phosphorylated serine and theronine residues of proteins (Pereira et al. 2011). There are several classes of phosphoprotein phosphatases, and the PPPs have been widely studied due to their role in regulation of cell processes, including gene expression, DNA replication, glycogen metabolism, and apoptosis, which are important in understanding diseases such as diabetes and cancer (Pereira et al. 2011). The particular gene whose expression levels was measured is homologous to phosphoprotein phosphatases of types 1 and 2A (PP1 and PP2A).

Phosphoprotein phosphatases play at least two roles in photosynthesis. In higher plants, phosphorylation and dephosphorylation are involved in the repair of the damaged D1 subunit in Photosystem II (Vener 2008). Nonetheless, green algae lack a phosphorylation-based pathway for D1 repair, and it has been that chloroplast-encoded heat shock protein HSP70B instead plays a role in D1 repair (Yokthongwattana et al. 2001). A second key role of phosphatases in photosynthesis is that of state transitions. State transitions are a mechanism by which an organism may balance the relative rates of Photosystem I and Photosystem II by regulating the excitation levels of the two photosystems (Minagawa 2011). Nonetheless, the phosphoprotein phosphatase involved in state transition is one of type PP2C and has been shown not to be inhibited by microcystins (Hammer et al. 1997; Minagawa 2011). Thus, the increased production of LHCBs cannot be explained by the inability to dephosphorylate LHCII complexes that migrated to PS I during a state transition, resulting in a need of *de novo* synthesis of LHCII complexes for PS II.

Nonetheless, phosphoprotein phosphatases of types 1 and 2A still have a variety of roles. Type 1 phosphatases (PP1) are involved in "synaptic transmission, gene expression, glycogen metabolism, RNA splicing, and cell-cycle progression" (Pereira et al. 2011). Type 2A phosphatases (PP2A) are implemented in "cell-cycle regulation, cell growth control, cytoskeleton dynamics, cell mobility, metabolism, transcription, translation, RNA splicing, DNA replication, apoptosis, inflammation, and differentiation" (Pereira et al. 2011). The wide variety of cell processes in which phosphatases are involved indicate the importance of this enzyme.

Substantial upregulation of mRNA corresponding to phosphoprotein phosphatase would be one effect of the possible presence of microcystins on the *Chlorella vulgaris* in the CC cultures. Likewise, expression levels of the gene are low in the Chl cultures at all four culture

conditions, which would not have the presence of microcystins due to the absence of the cyanobacteria. Nonetheless, other substances synthesized by cyanobacteria can have similar effects. For example, nodularins are also known to inhibit PP1 and PP2A (Honkanen et al. 1991; Wiegand and Pflugmacher 2005). An upregulation of gene expression for a PP2 and a concomitant decrease in PP2 protein activity has been observed in the clam *Corbicula fluminea* when exposed to the microcyctin MC-LR at a level of 5 µg/L (Martins et al. 2011). Furthermore, cyanobacteria of the species *Leptolyngbya boryana*, the species to which the cyanobacteria in the co-culture is most similar, are known to produce microcystins in a freshwater setting (Mohamed and Al Shehri 2010).

The effects of microcystin exposure to green algae have been documented in a number of studies. Exposure of *Scenedesmus quadricauda* to microcystin MC-LR at a level of $5x10^{-7}$ M resulted in a statistically significant ($p<0.05$) increase in chlorophyll a content per unit cell volume, with nearly twice the content 8 days after exposure. An increase in cell volume was statistically significant ($p<0.05$) at days 2 and 4 following exposure. Finally, microcystin exposure also resulted in larger chloroplasts (Sedmak and Eleršek 2005). Increased chlorophyll content and larger cell volume are consistent with the flow cytometry data for the CC cultures as compared to the Chl cultures.

One key characteristic of the CC cultures compared to the Chl cultures is the presence of a viscous exudate. A study on *Scenedesmus quadricauda* and *Chlorella vulgaris* documented the excretion of polysaccharides by algae exposed to MC-LR and crude microcystins, with increased polysaccharide production resulting from increasing toxin concentration (Mohamed 2008). The polysaccharides were determined to have antioxidant activity, with differing activity based on

their sulfate content. The purpose of the polysaccharides was to scavenge free radicals generated due to oxidative stress that was caused by the exposure to the microcystins.

4.5.9 Oil Globule Associated Protein

Expressed sequence tags containing sequences for oil globule asociated protein were found in 1 subtracted library for genes upregulated in the CC cultures. Expression levels were higher in the CC cultures compared to the Chl cultures for all 4 culture conditions at the 99.9% confidence level (Figure 4.22).

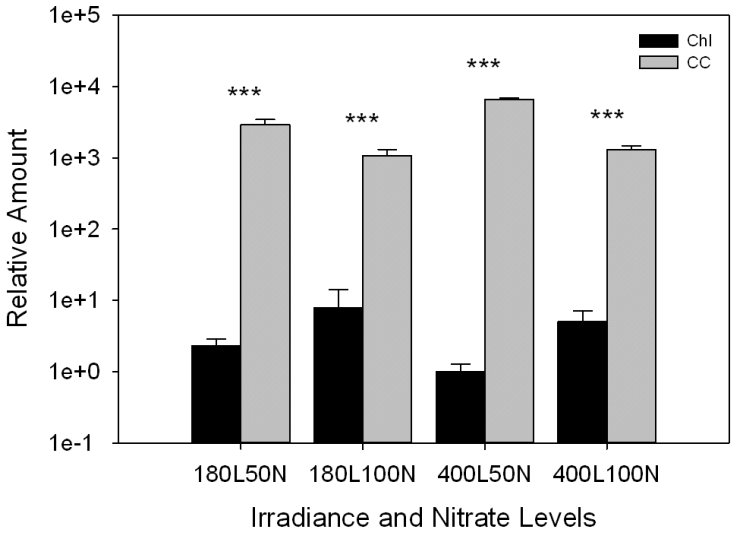


Figure 4.22: Gene expression of oil globule associated protein normalized to the lowest expression level among the 8 cultures. Values are mean ± S.E. from triplicates. Statistical significance of expression level difference between Chl and CC cultures at the same irradiance and nitrate level indicated by *** ($p<0.001$). P-values for effects of irradiance and nitrate on gene expression levels are 0.8874 and 0.6707, respectively.

For the 4 Chl cultures, neither the effects of irradiance ($p=0.6991$) nor nitrate level ($p=0.1320$) on expression level were significant. For the 4 CC cultures, the effect of irradiance was not significant ($p=0.3939$), but the effect of nitrate level was ($p=0.0022$). Across the 8 cultures, neither the effect of irradiance level ($p=0.8874$) nor the effect if nitrate level ($p=0.6707$) was significant. The effect of nitrate limitation on lipid synthesis in green algae has been widely

studied (Illman et al. 2000; Converti et al. 2009; Courchesne et al. 2009). Thus, upregulation of a gene associated with lipid storage would be consistent with the cultures having lower nitrate levels, as is the case for the oil globule associated protein, at least for the CC cultures.

The cultivation of microalgae for lipids involves both the need to maximize lipid production, but knowledge of how and why cells store lipids is also important. Since starch granules were known to form in some algae cells, researchers have become interested in the factors promoting the formation of cytoplasmic lipid bodies, such as in *Chlamydomonas reinhardtii* (Wang et al. 2009). Recent work has characterized proteins that are associated with the algal cytoplasmic lipid bodies, and these proteins have been termed oil globule associated proteins. Intracellular lipid droplets are found in organisms of all levels of complexity, and in algae they have been found to comprise as much as 86% of the cell mass in certain cases (Murphy 2011). A study on *Haematococcus pluvialis* found that the oil globule associated protein was barely present in cells until exposed to nitrogen depletion and high light conditions, which are known to promote enhanced lipid production in cells (Peled et al. 2011). The amino acid sequence of the oil globule associated protein (ascession no. HQ213938) was aligned with homologous sequences for other green algae of the order Volvocales, including *Chlamydomonas reinhardtii* (ascession no. XP_001697668), *Volvox carteri f. nagariensis* (ascession no. FD812477), and *Polytomella parva* (ascession no. EC718417). A study on major lipid droplet proteins in *Dunaliella* identified homologous proteins for *Dunaliella salina* (ascession no. JQ011390), *Dunaliella bardawil* (ascession no. JQ011391), and *Dunaliella parva* (ascession no. JQ011392) (Davidi et al. 2012). Expressed sequence tags for major lipid droplet proteins in the Chlorellales order have been identified for *Coccomyxa* sp. C-169 (ascession no. GW222322), *Chlorella variabilis* (ascession no. EFN52470), *Prototheca wickerhamii* (ascession no.

EC182117), and *Micromonas pusilla* CCMP1545 (ascession no. XP_003057303). Nonetheless, orthologs to the algal lipid droplet proteins appear to be confined to green algae (Moellering and Benning 2009; Murphy 2011).

While the role of the oil globule associated protein is unknown, the identification of no known catalytic activity for the protein suggests that the lipid droplet protein may have a structural role (Moellering and Benning 2009). A hypothesized role is preventing fusion of lipid droplets, resulting in a larger surface area-to-volume ratio to better allow enzymes to access TAGs in the lipid droplets (Moellering and Benning 2009). Finally, the presence of GTPases, ADP ribosylation factor 1 (ARF1), and other proteins related to vesicular trafficking that are found in proteins extracted from animal lipid droplets provides support that lipid droplets in *C. reinhardtii* may play similar roles to those of higher organisms (Murphy 2011). Genes present in the subtracted libraries included two GTPases, ADP ribosylation factor, and a multivesicular body protein, which are related to vesicular trafficking. More work is expected to be done on oil globule associated proteins in green algae, as a better understanding of their role in cytoplasmic lipid storage is likely to be beneficial to enhanced lipid production in microalgae.

4.5.10 Discussion of Cyanobacterial Compounds

A number of cyanobacterial compounds have been shown to inhibit growth of *Chlorella pyrenoidosa* (Ikawa et al. 2001). These include geosmin, β-cyclocitral, α-ionone, β-ionone, and geranylacetone. Compounds in the study that did not cause growth inhibition were geranylgeraniol and dimethyl disulfide. However, there is also evidence that microcystins do not inhibit all photosynthetic organisms. Duckweed (*Lemna gibba*) did not show any effect from microcystin-LR (LeBlanc et al. 2005), although both PSII and growth are inhibited in *Lemna minor* by fischerellin A (Berry 2008) .

Thus, a number of different cyanobacteria-synthesized substances are known to create effects in green algae and other plants similar to those observed in the CC cultures. While testing the media of the CC cultures for these may prove difficult, partly due to the low levels at which these compounds might be present, identification of one or more of these substances would provide a possible explanation for the gene expression patterns in the Chl cultures compared to the CC cultures.

4.5.11 Conclusion

An examination of the gene expression patterns for the 9 genes which were studied as a whole reveals several patterns. First, no gene displayed significantly significant expression levels across all 8 cultures due to the effects of irradiance or nitrate level was that for oil globule associated protein. On the other hand, every gene showed a statistically significant difference in expression level at at least the 99% confidence level between the Chl and CC cultures grown at the same irradiance and nitrate level. This trend shows that the effect of co-culturing is extremely significant, whereas the effects of irradiance and nitrate level on gene expression were less significant.

Second, a variety of cyanobacterial-produced substances can produce the effects observed in the gene expression data. Microcystins and nodularins, inhibitors of phosphoprotein phosphatases, have already been discussed. Fischerellins, cyanobactrin, and tellimagrandin II are all known to inhibit PSII (Weir et al. 2004), which could explain the upregulation of light-harvesting chlorophyll-binding protein in the CC cultures. Inhibition of PSII could also trigger stress due to reactive oxygen species, which would explain upregulation of heat shock proteins as well.

CHAPTER 5: CONCLUSIONS AND FUTURE WORK

5.1 General Discussion and Conclusions

The goal of this work was to determine why the *Chlorella vulgaris* grew better in the co-culture than in the monoculture, resulting in larger cells, increased biomass levels, and increased lipid productivity. The co-culture and *Chlorella* monocultures were cultivated at two nitrate levels and two irradiance levels in a 2x2 factorial experiment. Differential gene expression experiments on the *Chlorella vulgaris* in the co-culture with cyanobacteria *Leptolyngbya* sp. and in a monoculture were performed using suppression subtractive hybridization, and expression levels of actin and 9 selected genes were performed using quantitative PCR.

Data from the subtracted libraries and the qPCR, as well as observations of the co-culture *Chlorella vulgaris* using flow cytometry, reveal that many aspects of the co-culture can be explained by the possible production of microcystins or another cyanobacteria-synthesized substance by the *Leptolyngbya* sp. First, the presence of larger cells with increased chlorophyll content resulting from microcystin treatment has been documented for green alga *Scenedesmus quadricauda* (Sedmak and Eleršek 2005). Upregulation of PSI reaction center subunit XI in the and light-harvesting chlorophyll-binding protein in the CC cultures as compared to the Chl cultures support the increased production of chlorophyll observed by flow cytometry for the co-culture sample. Second, *Chlorella vulgaris* has been found to synthesize polysaccharides with antioxidant properties in response to microcystin exposure (Mohamed 2008). Upregulation of the gene for 60S ribosomal protein L23a, related to the production of proteins for insertion into membranes and secretion might be related to the secretion of the polysaccharides and possibly other substances such as proteins. Third, levels of mRNA transcripts for phosphoprotein phosphatase, a protein known to be targeted by both microcystins and nodularins, was upregulated in the CC. Fourth, upregulation of a heat shock protein suggests the presence of

reactive oxygen species (ROS), which could be the result of a variety of cyanobacterial substances that inhibit PSII, including fischerellins, cyanobactrin, and tellimagrandin II. Nonetheless, it is not known whether the *Leptolyngbya* sp. in the CC cultures produces any of these substances. Furthermore, gene expression data does not support the upregulation of the heat shock protein due to photinhibition, for which higher levels of upregulation would be expected for the higher irradiance level, which is not the case. Finally, the inhibition of the phosphoprotein photphatases by microcystins and nodularins and effects on cell processes of cell-cycle regulation, cell growth control, and cytoskeleton dynamics may explain why cell counts in the CC cultures were far below those in the Chl cultures.

An additional key finding is the identification of an oil globule associated protein in a subtracted library, and verified upregulation of mRNA coding for the protein in the CC cultures. The protein is a member of lipid droplet proteins previously identified for a number of green algae in the orders Volvocales and Chlorellales (Peled et al. 2011; Davidi et al. 2012).

One interesting finding is that among the 2x2 factorial culture conditions of starting nitrate levels corresponding to 50% and 100% of the nitrate levels of Bold's Basal Medium and the irradiance levels of 180 µmol/m^2-sec and 400 µmol/m^2-sec, the gene expression patterns did not vary significantly. The main variation in gene expression, instead, was between monoculture *Chlorella* and co-culture *Chlorella*, which suggests that the same phenomenon explains gene expression differences in the co-culture versus the monoculture at a variety of culture conditions.

5.2 Future Work

5.2.1 Testing for Cyanobacteria-Produced Substances

A key step in future work will be to test for the presence of microcystins or other cyanobacterial-produced substances such as nodularins and fischerellins, in the culture medium.

While cyanobacteria of species *Leptolyngbya boryana*, the species to which the cyanobacteria in the co-culture is most similar, have been documented to product microcystins, the microcystins might not be present at levels easily detectable (Mohamed and Al Shehri 2010). Additionally, *Leptolyngbya* may produce other substances—possibly previously unknown ones—that could have an effect on the *Chlorella vulgaris*. Finally, the *Chlorella* biomass could also be tested for the possible accumulation of the microcystins.

An additional set of experiments can be performed to gauge the effect of exogenously applied microcystins and other cyanobacteria-produced substances on the monoculture *Chlorella vulgaris*. A good experimental framework for this investigation will be that used to test if a particular process resulted due to the action of a plant hormone (Bradley 1991). It will be interesting to see if the treatment of the Chlorella monoculture with microcystins but without the presence of the *Leptolyngbya* sp. will produce any of the effects of the co-culture, such as increased cell volume, increased chlorophyll content, a viscous exudates, the ability to resist invasion by other microorganism, or enhanced lipid productivity.

5.2.2 Collection of RNA During Different Growth Phases

The RNA collected for the current study was collected during the late exponential growth phase. Collection of RNA during different growth phases, especially the early exponential phase, for further gene expression studies might yield additional insight into the differential gene expression of the co-culture *Chlorella* versus the monoculture *Chlorella*. In particular, the effect of the co-culture environment on the rapid growth of the cultures and the corresponding gene expression patterns could be examined and compared.

5.2.3 Variance of Light Regimes

Since a large number of the experiments on green algae use light:dark cycles, experiments using similar cycling should be done. A comparison of gene expression for

photosystem genes for light:dark cycles or for cultures grown at a lower irradiance will provide further support that gene expression showing oxidative stress, such as upregulated expression of heat shock proteins, is not due to photoinhibition. In addition, use of light:dark cycles will better simulate outdoor lighting conditions that would be used for large-scale microalgal cultures.

In addition, experiments should also be performed at higher irradiance levels known to cause photodamage. If *Leptolyngbya* sp. does indeed produce a substance that is affecting the growth of the *Chlorella vulgaris*, photodamage would likely selectively hinder the growth of the cyanobacteria compared to the *Chlorella vulgaris*. The response of the co-culture to conditions which partially inhibit the growth of the *Leptolyngbya* sp. might provide additional insight into the interaction of the two species.

5.2.4 Co-culture Exudate

Finally, the viscous exudate present in the co-culture media needs to be examined further. Tests similar to those for the polysaccharides found from *Chlorella vulgaris* and *Scenedesmus quadricauda* (Mohamed 2008) could be performed. If the exudate does indeed contain a polysaccharide with antioxidant properties, further investigation could be done to determine its structure. In addition, a liquid-liquid extraction procedure could be developed to purify it from the culture media. Finally, if the polysaccharide does contain sulfate as in the ones collected from microcystin-treated *Chlorella* and *Scenedesmus* cultures (Mohamed 2008), an isotopic study using ^{35}S in the media might elucidate possible metabolic pathways resulting in production of the polysaccharide, providing targets for metabolic engineering for production of this possibly high-value product.

5.2.5 Conclusion

The discovery of a possible explanation for many of the aspects of the *Chlorella vulgaris*/*Leptolyngbya* sp. co-culture compared to the *Chlorella vulgaris* monoculture should

provide much opportunity for further studies. Insights gained from further investigation could possibly yield a better understanding of microalgae-cyanobacteria co-cultures in general and production of a variety of bioproducts from algae, including but not limited to lipids.

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APPENDIX A: DETAILED DESCRIPTIONS OF EXPERIMENTAL METHODS

A.1 Dry Biomass Determination

Dry biomass measurements of the culture were taken according to Environmental Engineering/Water Quality Laboratory Standard Operating Procedure (EiEL/Water Quality Laboratory SOP) PA 200, Total Suspended Solids Freshwater Algae Matrix, which is based on Standard Methods for the Examination of Waste and Wasterwater 2540D (APHA 1999). Whatman GF/C filters (Piscataway, New Jersey), glass fiber filters with a pore size of 1.2 µm, were rinsed with deionized water, placed in an aluminum pan, and heated in a 550ºC furnace for 20 minutes. Afterward, the filters were cooled in a dessicator. Before use, the filters were weighed on an analytical balance and their masses recorded. The GF/C filter was dampened with deionized water and put into the filter funnel. Five or 10 ml of algae culture was mixed by vortexing, measured in a pipet, and filtered through the filter using a vacuum pump connected to a filter flask. The filter funnel was rinsed three times with deionized water to make sure the entire algae sample had reached the filter and to rinse through particles smaller than the pore size of the filter. Following filtration, the samples had an appearance as shown in Figure 3.2. The filters were then dried for 1-3 hours at 65ºC in a furnace. After cooling in a dessicator, the filters were weighed on an analytical balance. Blanks were prepared with the culture medium without algae. The dry biomass of the algae was determined by subtracting the mass of the blank from the mass of the filter with the algae on it. Dry biomass measurements were taken for algae samples collected at four points for each of the eight algae cultures grown: when the culture was inoculated, during the early exponential phase, when the culture was harvested for RNA, and when the culture had reached the stationary growth phase.

A.2 Folch Extraction of Lipids

Lipids were extracted from each culture using algae samples collected at two points for each of the eight algae cultures grown: when the culture was harvested for RNA and when the culture had reached the stationary phase of growth. Lipids were extracted using a modified Folch lipid extraction protocol (Folch et al. 1957). The extractions were performed in triplicate for each algae sample.

Figure A.1: Evaporation of solvent using a rotary evaporator.

Eighty ml samples of culture were frozen at -20ºC upon collection. Prior to lipid extraction, the samples were thawed at 4ºC. Samples were homogenized, and 50 ml was pipetted into a 50 ml polypropylene plug seal centrifuge tube and pelleted by centrifugation at 3,000x*g* for 10 minutes at 4ºC. The supernatant was decanted with care taken not to disrupt the algae pellet. An additional 30 ml of culture was added to the same 50 ml centrifuge tube, and the

centrifugation was repeated. Once again, the supernatant was decanted with care taken not to disrupt the algae pellet. Thirty-five ml of 2:1 v/v $CHCl_3$: CH_3OH was added to each tube, and the tube was capped and wrapped in aluminum foil to protect the chloroform from light and for safety in case of tube leakage.

The tube was shaken for 20 minutes at 100 rpm and 29ºC in an Innova 4300 Incubator Shaker (New Brunswick Scientific, Enfield, Connecticut). Seven ml, one-fifth the volume of the $CHCl_3$:CH_3OH solvent, of 0.9% w/v NaCl solution in deionized water was added to the tube, which was capped and shaken vigorously for 5 seconds to mix the phases. To separate the phases, the tube was centrifuged for 10 minutes at 1460x*g* at room temperature. Following centrifugation, a transparent aqueous layer, a dark green biomass interphase, and a transparent green organic layer were visible. The aqueous layer was carefully removed using a Pasteur pipet and was discarded. Using a separate pipet, the biomass layer was carefully penetrated, and all of the organic phase was transferred to a 250 ml borosilicate glass flat-bottom boiling flask, taking care not to introduce any of the biomass layer into the flask. The $CHCl_3$ was evaporated using a rotary evaporator (Figure A.1) until no liquid remained visible.

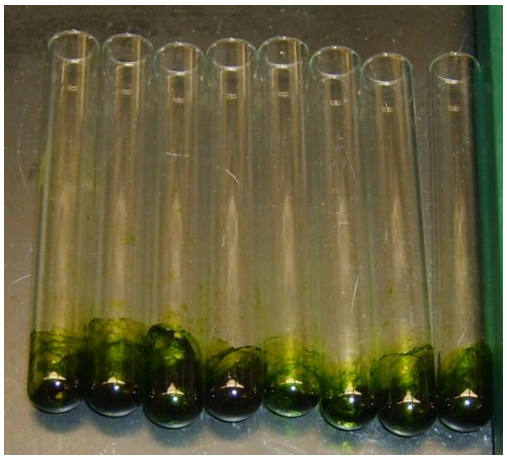

Figure A.2: Eight glass test tubes used in Folch lipid extraction, following aspiration of the solvent with nitrogen gas.

The residue, which contained the lipids, was dissolved in a small volume of the 2:1 v/v $CHCl_3$: CH_3OH solution and pipetted into a glass test tube that had been preweighed on an analytical balance. Finally, the remaining solvent was evaporated by aspiration with N_2 gas, leaving a dry residue of the lipids; the appearance of the test tubes with the dry lipid residue is shown in Figure A.2.

The lipid mass was computed by subtracting the original mass of the test tube from the mass of the tube with the lipids, and the lipids as percent of dry mass were computed from the dry biomass measurements taken for the samples.

A.3 Determination of RNA Quantity and Quality by UV Spectroscopy

Total RNA samples were analyzed to determine concentration and test for impurities using UV spectroscopy. Two µl of each total RNA sample was placed into a nanoVette (Beckman Coulter, Brea, California) spectrophotometer cuvette with a 0.2 mm pathlength in a DU730 Life Science UV/Vis Spectrophotometer (Beckman Coulter, Brea, California). After blanking the device with the TE buffer, pH 7.5, that the RNA was dissolved in, absorbances at 260 nm, 280 nm, and 230 nm were taken. The 260 nm absorbance, or A_{260}, measures the concentration of nucleic acid in the sample, with an A_{260} of 1 corresponding to a nucleic acid concentration of 40 µg/ml. The A_{260}/A_{280} measures the degree of protein contamination, since protein absorbs at 280 nm (hence lowering the ratio), with an ideal ratio of approximately 2.0. Finally the A_{260}/A_{230} measures for organic compound and polysaccharide contamination (both of which lower the ratio due to their absorbance at 230 nm), with ideal ratios for RNA in the range of 2.0-2.4 (Farrell 2009).

Following UV spectroscopy, total RNA samples were stored in aliquots at -80ºC to reduce the number of freeze-thaw cycles to which a particular sample would be subjected.

A.3 DNase Treatment of RNA Samples and RNA Cleanup

Following quantification and sample quality analysis, total RNA samples were treated to remove contaminating DNA. Five µg of each total RNA samples was diluted in water to a final volume of 50 µl. Deoxyribonuclease (DNase) treatment was performed using the TURBO DNA-*free*™ kit (Ambion, Grand Island, New York) according to the manufacturer's protocol. One µl (2 U/µl) of TURBO DNase and 5 µl of 10X TURBO DNase buffer was added to the diluted RNA sample and mixed by vortexing. The sample was incubated at 37ºC for 30 minutes in a water bath. Afterward, 5 µl of DNase Inactivation Reagent was added to each sample, and the sample was vortexed. After incubation at room temperature for 2 minutes with gentle mixing, the sample was centrifuged at 10,000x*g* for 1.5 minutes to pellet the inactivation reagent. The supernatant, containing the RNA sample, was moved to a new tube, and water was added to a total volume of 100 µl per sample.

The RNA samples were further purified up and concentrated using RNA Clean & Concentrator™-5 columns (Zymo Research, Irvine, California) according to the manufacturer's protocol. Two-hundred µl of RNA Binding Buffer and 150 µl of absolute ethanol were added to each RNA sample that had been treated with DNase, after which the tubes were vortexed. The mixture was transferred to a spin column and centrifuged at 12,000x*g* for 1 minute; the flow-through was discarded. Four-hundred µl of RNA Prep Buffer was added to each column, after which it was centrifuged at 12,000x*g* for 1 minutes and the flow-through discarded. Finally, the column was washed twice, first with 800 µl and then with 400 µl of RNA Wash Buffer. Following addition of wash buffer in each step, the column was centrifuged at 12,000x*g* for 1 minute, and the flow-through was discarded. Then the column was centrifuged for 12,000x*g* for 2 minutes to elute any residual fluid from the washing steps. Finally, the RNA samples was

eluted by added 6 µl of water directly to the column matrix, letting the column stand for 1 minute at room temperature, and centrifuging the column 10,000x*g* for 30 seconds.

One µl of each sample following DNase treatment and cleanup was analyzed by UV spectroscopy. As done previously, the absorbances at 260 nm, 280 nm, and 230 nm (A_{260}, A_{280}, and A_{230}, respectively) were taken to analyze the RNA concentration and test for the presence of proteins and polysaccharides in the samples. One µg of each RNA sample was diluted in water to a total volume of 3.5 µl and stored at -80ºC until cDNA synthesis was performed. Four-hundred nl of each RNA sample immediately used for reverse transcription, and the remainder of each sample was diluted by the addition of 9 µl of water and frozen at -20ºC until to use in gel electrophoresis after reverse transcription was performed.

A.4 Sample Quality Assessment by Reverse Transcription

The quality of the RNA samples was further tested through reverse transcription, the synthesis of DNA from RNA. Reverse transcription was performed using the SMARTScribe™ Reverse Transcriptase enzyme (Clontech, Mountain View, California) according to the manufacturer's protocol. Four-hundred ng of each RNA sample was diluted in water to a total volume of 4 µl. One µl of oligo$(dT)_{18}$ primer (20 µM) was added to the diluted RNA sample. The RNA and primer mixture was heated at 72ºC for 3 minutes to denature the sample and then immediately cooled on ice. Then 2 µl of 5X First-Strand Buffer, 1 µl of dNTP Mix (10 µM each dNTP) and 1 µl 20 mM dithiothreitol (DTT) were added, and the tube contents were mixed by pipetting up and down. Finally, 1 µl of SMARTScribt RT enzyme (100 U/µl) was added, and the tube contents were mixed again. The reverse transcription reactions were incubated at 42ºC for 90 minutes in a DNA Engine® PTC-200 thermal cycler (Bio-Rad, Hercules, California). Afterward, the reaction was terminated by heating the tubes to 70ºC for 15 minutes.

The RNA samples before and after reverse transcription were visualized using gel electrophoresis. A 1.2% agarose gel was prepared with 55 ml of 0.5X TBE buffer. 4.5 µl of ethidium bromide (EtBr) solution (10 mg/ml) was added to the molten agarose before the gel was poured. The gel was run in 0.5X TBE buffer for 1 hour at 80 V. The gel was visualized using a BioSpectrum®500 MultiSpectrum Imaging System (UVP, Upland, California) with a Benchtop 2UV™ Transilluminator (UVP, Upland, California). The gel was transilluminated with 302 nm light, and images were captured and analyzed using the VisionWorks™LS Image Acquision and Analysis Software, version 6.5.2 (UVP, Upland, California).

A.5 SMARTer PCR cDNA Synthesis

Total RNA samples were prepared for suppression subtractive hybridization through cDNA synthesis and amplification. Samples were removed from the -80ºC freezer and placed on ice. Complementary DNA (cDNA) was synthesized from total RNA using the SMARTer™ PCR cDNA Synthesis Kit (Clontech, Mountain View, California) according to the manufacturer's protocol (Clontech Laboratories 2011b), following a version of the protocol specific to samples later used in the PCR-Select™ cDNA Subtraction Kit (Clontech, Mountain View, California). The protocol for the SMARTer kit consisted of the following steps: first-strand cDNA synthesis, cDNA amplification by PCR, column chromatography, RsaI digestion, and purification of digested DNA.

First-strand cDNA was synthesized according to the protocol using 1 µg of each total RNA sample. Reverse transcription was performed using the 3' SMART CDS Primer II A and SMARTer IIA Oligonucleotide with the SMARTScribe Reverse Transcriptase enzyme. Each 1 µg total RNA sample was diluted to a volume of 3.5 µl in water in a 200 µl PCR tube, and 1 µl of 3' SMART CDS Primer II A (12.5 µM) was added. The tubes were incubated in the thermal cycler at 72ºC for 3 minutes, followed by incubation at 42ºC for 2 minutes. A reverse

transcription master mix was prepared at room temperature. The master mix consisted of 2 µl 5X First-Strand Buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 30 mM $MgCl_2$), 0.25 µl DTT (100 mM), 1 µl dNTP Mix (10 µM of each dNTP), 1 µl SMARTer II A Oligonucleotide (12 µM), 0.25 RNase Inhibitor, and 1 µl SMARTScribe Reverse Transcriptase (100 U). Five-and-a-half µl of master mix was added to each sample of total RNA and the 3' SMART CDS Primer II A, for a total reaction volume of 10 µl. The reactions were incubated for 90 minutes at 42ºC, after which each 10 µl reaction was diluted by adding 40 µl of TE buffer (10 mM Tris [pH 8.0], 0.1 mM EDTA). The diluted reverse transcription products were stored at -20ºC.

For each sample, first-strand cDNA was amplified in 300 µl of PCR reactions. Ninety µl of PCR Master Mix was prepared per sample. The PCR Master Mix consisted of 74 µl deionized water, 10 µl 10X Advantage 2 PCR Buffer (400 mM Tricine-KOH [pH 8.7 at 25ºC], 150 mM KOAc, 35 mM $Mg(OAc)_2$, 37.5 µg/µl BSA, 0.05% Tween 20, 0.05% Nonidet-P40), 2 µl dNTP Mix (10 µM each dNTP), 2 µl 5' PCR Primer II A (12 µM), and 2 µl 50X Advantage 2 Polymerase Mix (TITANIUM Taq DNA Polymerase, a small amount of proofreading polymerase, and 1.1 µg/µl TaqStart antibody in a solution of 50% glycerol, 15 mM Tris-HCl [pH 8.0], 75 mM KCl, 0.05 mM EDTA). Thirty µl of each diluted first-strand cDNA reactions was mixed with 270 µl of PCR Master Mix, and the mixture was divided equally into three 200 µl PCR tubes for thermal cycling. All tubes were subjected to 95ºC for 1 minute and 15 cycles of 95ºC for 15 seconds (denaturation), 65ºC for 30 seconds (annealing), and 68ºC for 6 minutes. Thirty µl was removed from one of the three tubes for each sample, and the remaining 270 µl were stored at 4ºC. The 30 µl sample was subjected to 12 additional cycles of PCR, with 5 µl being removed after each 3 cycles. For each sample, a 5 µl aliquot was collected after 15, 18, 21, 24, and 27 cycles of PCR, respectively. The samples were compared on a 1.2% agarose gel

to determine the optimal number of PCR cycles to amplify the cDNA without changing the expression profile of the genes in the sample. Following determination of the optimal number of cycles, each sample was removed from 4ºC and subjected to the optimal number of cycles. A 5 µl aliquot of each the final PCR product for each sample was visualized on 1.2% agarose/EtBr gel to confirm that PCR was successful. PCR products were stored at -20ºC or used immediately in column chromatography.

The PCR products were subjected to column chromatography according to the manufacturer's protocol. Each PCR product reaction mixture was mixed with phenol:chloroform:isoamyl alcohol 25:24:1 (pH 8.0) and centrifuged at 14,000 rpm for 10 minutes, which removed the RNA that had not been reverse transcribed and the DNA polymerase enzyme. The aqueous phase was concentrated to a smaller volume by butanol extraction. Seven-hundred µl of sec-butanol was added to the aqueous phase, the mixture was vortexed, and the solution was centrifuged for 1 minute at 14,000 rpm. The lower or aqueous phase was collected and put in a new 1.5 ml tube, after which the butanol phase was discarded. Water was added if necessary to bring the volume of the aqueous phase to 40-70 µl. The mixture was fed onto a CHROMA SPIN+TE-1000 column that had been equilibrated with 1X TNE buffer (10 mM Tris-HCl [pH 8], 10 mM NaCl, 0.1 mM EDTA). The size-exclusion chromatography process allowed shorter nucleic acid fragments to elute first, and the later fractions, corresponding to longer fragments, were collected. A 1.2% agarose/EtBr was run to visualize the column chromatography eluent and verify that the PCR present was present.

The cDNA samples were then digested using the restriction enzyme RsaI. To each sample following column chromatography, 36 µl of 10X RsaI restriction buffer (100 mM Bis Tris Propane-HCl [pH 7.0], 100 mM $MgCl_2$, 1 mM DTT) and 1.5 µl of RsaI (10 U/µl) were

added. Digestion was performed at 37ºC in a water bath for 3 hours, after which the digestion reaction was terminated by the addition of 8 µl of 0.5 mM EDTA. The digestion resulted in shorter, blunt-ended fragments that are used in the suppression subtraction hybridization procedure. Successful digestion, resulting in shorter cDNA fragments, was verified using a 1.2% agarose/EtBr gel.

Finally, samples were purified to remove the RsaI enzyme and salts used in the RsaI buffer. The digested cDNA was purified using the NucleoTraP®CR silica matrix-based purification system (Macherey-Nagel, Düren, Germany) according to the Clontech SMARTer kit protocol. The purified cDNA was precipitated by ammonium acetate and ethanol and then washed once with 80% ethanol. The cDNA pellet was air-dried and dissolved in 6.7 µl of 1X TNE buffer (10 mM Tris-HCl [pH 8], 10 mM NaCl, 0.1 mM EDTA). The concentration was determined using UV spectroscopy, and the cDNA was diluted to a concentration of 300 ng/µl if it exceeded it using TNE buffer. Samples were stored at -20ºC.

A.6 Suppression Subtraction Hybridization

Previously stored cDNA samples following RsaI digestion were removed from the -20ºC freezer. One µl of each cDNA sample was diluted with 5 µl of sterile water, and 2 µl of the diluted cDNA was used in each ligation reaction. A ligation master mix was prepared, with 3 µl of sterile water, 2 µl of 5X Ligation Buffer (250 mM Tris-HCl [pH 7.8], 50 mM $MgCl_2$, 10 mM DTT, and 0.25 mg/ml BSA), and 1 µl of T4 DNA ligase (400 U/µl). The ligation reactions, consisting of 2 µl of diluted tester cDNA, 2 µl of either Adapter 1 or Adapter 2R (10 µM), and 6 µl of the ligation master mix, were set up in 200 µl PCR tubes. A third reaction, called the unsubtracted tester control, was performed using both adapters. The unsubtracted tester control reaction consisted of 2 µl of the Adapter 1 ligation reaction and 2 µl of the Adapter 2R ligation reaction. The ligation reactions were performed using T4 DNA ligase overnight at 16ºC in a

thermal cycler, after which the reaction was stopped by the addition of 1 µl of EDTA/Glycogen Mix. One µl of the unsubtracted tester control was diluted into 1 ml of water and stored at -20ºC until later use in PCR.

The adapter-ligated tester samples were now prepared for the first hybridization. One µl of 4X Hybridization Buffer was combined with 1.5 µl of RsaI-digested driver cDNA and 1.5 µl of one of the adapter-ligated tester samples. Although the same volume of driver and tester cDNA was combined, the driver cDNA was 10 times more concentrated at this point in the protocol due to the dilution of the tester cDNA during ligation. The samples were overlaid with 10 µl of mineral oil to prevent evaporation, after which they were denatured at 98ºC for 1.5 minute and incubated for 8 hours at 68ºC in the thermal cycler.

For the second hybridization, freshly denatured driver cDNA was prepared, and the fresh driver cDNA and both samples from the first hybridization were combined. One µl of driver cDNA, 1 µl of 4X Hybridization Buffer (composition unknown), and 2 µl of water were combined. One µl of this mixture was placed in a new PCR tube and overlaid with 4 µl of mineral oil. The driver cDNA was denatured at 98ºC for 1.5 minutes in the thermal cycler. The denatured driver cDNA and the two samples from the first hybridization were then combined simultaneously. A micropipettor was set to 20 µl with a 20-200 µl aerosol tip. The freshly denatured driver sample and mineral oil were drawn into the tip. Then one of the samples from the first hybridization was drawn into the tip, leaving an air space in the pipet tip. Finally, the entire contents of the pipet tip were eluted into the other sample from the first hybridization, allowing the two different first hybridization samples and the freshly denatured driver to mix simultaneously. The tube contents were mixed by pipetting up and down, and the tube was briefly centrifuged to bring the mineral oil layer to the top. The second hybridization was

performed at 68ºC overnight in the thermal cycler. Upon completion of the second hybridization, 200 µl of dilution buffer (20 mM HEPES [pH 6.6], 20 mM NaCl, 0.2 mM EDTA [pH 8.0], adjusted to a final pH of 8.3) was added to each sample, and the samples were heated at 68ºC for 7 minutes in the thermal cycler. The samples were then stored at -20ºC.

Following the second hybridization, two rounds of PCR were performed. The PCR amplified the differentially expressed cDNAs exponentially and reduced background. For primary PCR, a master mix was prepared. For each reaction, the master mix contained 19.5 µl sterile water, 2.5 µl 10X PCR reaction buffer, 0.5 µl dNTP Mix (10 mM), 1.0 µl PCR Primer 1 (10 µM), and 0.5 µl 59X Advantage cDNA Polymerase Mix. Twenty-four µl of primary PCR master mix was added to 1 µl of template DNA in a 200 µl PCR tube for each primary PCR reaction. For each subtracted library, two primary PCR reactions were performed, one with 1 µl of the diluted second hybridization product as the template and the other with 1 µl of the unsubtracted tester control as the template. The reactions were incubated at 75ºC in the thermal cycler for 5 minutes to extend the adapters, and then they were subjected to 27 cycles of PCR according to the following: 94ºC for 30 seconds (denaturation step), 66ºC for 30 seconds (annealing step), and 72ºC for 1.5 minutes (elongation step). Eight µl from each primary PCR reaction was visualized on a 2.0% agarose/EtBr gel and visualized. In the case that a PCR product was not visible after 27 cycles, the PCR reaction was subjected to 3 additional cycles. Following successful completion of primary PCR, 3 µl of the PCR product from each reaction was diluted with 27 µl of water in a new 200 µl PCR tube.

Secondary PCR was performed to further amplify the differentially expressed genes and reduce background. For secondary PCR, a master mix was also prepared. For each reaction, the master mix contained 18.5 µl sterile water, 2.5 µl 10X PCR reaction buffer, 1.0 µl Nested PCR

primer 1 (10 µM), 1.0 µl Nested PCR primer 2R (10 µM), 0.5 µl dNTP Mix (10 mM), and 0.5 µl 59X Advantage cDNA Polymerase Mix. Twenty-four µl of primary PCR master mix was added to 1 µl of template DNA in a 200 µl PCR tube for each primary PCR reaction. For secondary PCR, the template was the diluted primary PCR products. The reactions were subjected to 12 cycles of PCR according to the following: 94ºC for 30 seconds (denaturation step), 68ºC for 30 seconds (annealing step), and 72ºC for 1.5 minutes (elongation step). Eight µl from each primary PCR reaction was visualized on a 2.0% agarose/EtBr gel and visualized. The secondary PCR products were frozen at -20ºC.

A.7 Subcloning of Expressed Sequence Tags (ESTs) in *E. coli*

Following secondary PCR in the suppression subtractive hybridization, the PCR products, representing expressed sequence tags (ESTs) of differentially expressed genes, were prepared for subcloning into *E. coli.*

A.7.1 Silica Purification and Adenylation of SSH Products

Fifty-five µl of TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA) were added to 45 µl of secondary PCR products from each reaction, and the mixture was transferred to a 1.5 ml microfuge tube. The PCR products were purified using the NucleoTraP®CR silica matrix-based purification system (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. In particular, primers were removed in the process because they would preferentially ligate into the *E. coli* cloning vector compared to the SSH products, due to the shorter length of the primers compared to the SSH products.

The silica-purified cDNA was visualized on a 1.5% agarose/EtBr gel. The DNA concentration in the silica-purified eluent was estimated by comparing the band intensities to known band intensities of a GeneRuler™ 1kb Plus DNA ladder (Fermantas, Glen Burnie, Maryland).

For successful ligation into the *E. coli* cloning vector, the cDNA must have an overhanging adenine at the 3' end of the molecule, which is commonly called an A-tail. Because the Advantage 2 Polymerase Mix contains a proofreading polymerase that degrades the overhanging A-tail, the cDNA was adenylated in a separate reaction using a *Taq* polymerase that does not degrade the A-tail. Taq Full Hot Start DNA Polymerase (Clontech, Mountain View, California) was used for the A-tailing reaction, which was performed according to a protocol in the Technical Manual for the pGEM®-T and pGEM®-T Easy Vector Systems (Promega, Madison, Wisconsin). Seven µl of purified PCR product was combined with 1 µl of 10X Taq Full PCR Buffer (200 mM Tris-HCl [pH 8.5], 500 mM KCl, 20 mM $MgCl_2$, 0.1% Tween 20), 0.2 µl dATP (10 mM), 1 µl Taq Full Hot Start Polymerase (5 U/µl), and 0.8 µl water in a 200 µl PCR tube. The enzyme was activated and the DNA denatured by 3 minutes at 94ºC, and the A-tailing was performed at 72ºC for 30 minutes. The product was stored at -20ºC.

A.7.2 Ligation and Transformation of SSH Products

Following adenylation, the SSH products were ligated into the pGEM®-T Easy Vector (Promega, Madison, Wisconsin) according to the manufacturer's protocol. Ligation reactions were set up for each of the eight SSH libraries in 200 µl PCR tubes with 5 µl 2X Rapid Ligation Buffer (60 mM Tris-HCl [pH 7.8], 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 1-% polyethylene glycol [MW8000, ACS Grade]), 1 µl (50 ng) pGEM®-T Easy Vector, 2 µl of A-tailed PCR product, 1 µl of T4 DNA Ligase (3 Weiss units/µl), and 1 µl of water. A positive control was prepared using Control Insert DNA, and a background control was prepared without insert DNA. The ligation reactions were incubated overnight at 4ºC to maximize the number of vectors into which the SSH products were inserted. The remaining part of each ligation reaction was stored at -20ºC.

Before transforming the *E. coli* cells with the cloning vector, LB plates with ampicillin/ IPTG/X-Gal were prepared. LB was prepared by dissolving 14 g of tryptone, 7 g of yeast extract, and 7 g of NaCl in 1.4 L total volume of water in a 2 L bottle. The pH was adjusted to 7.0 through the addition of NaOH, and 21 g of agar was added. The LB agar medium was autoclaved and allowed to cool to 50ºC in a water bath, after which ampicillin was added to a final concentration of 100 µg/ml. The agar media was poured into Petri dishes, allowed to cool, and stored at 4ºC until use. On the day of the *E. coli* ligation, the plates were warmed to room temperature. One-hundred µl of ChromoMax IPTG/X-Gal solution (Thermo Fisher Scientific, Waltham, Massachusetts) was spread over each plate, and the plates were allowed to heat to 37ºC and dry for an hour.

The *E. coli* transformation was then performed according to the Promega protocol. Two µl of each ligation reaction was added to a 1.5 ml microfuge tube on ice. Tubes of frozen JM109 High Efficiency Competent Cells (Promega, Madison, Wisconsin) were removed from the -80ºC freezer and placed in an ice bath for approximately 5 minutes until thawed. The cells were mixed by flicking the tube, and 50 µl of the cell suspension was added to each tube with the ligation reaction product. The tubes were mixed by flicking and placed on ice for 20 minutes. Transformation was performed by heat shock; the tubes were placed in a water bath at 42ºC for 45 seconds. After the heat shock, the tubes were returned to ice for 2 minutes. Nine-hundred and fifty µl of SOC medium (100 ml total volume containing 2.0 g tryptone, 0.5 g yeast extract, 1 ml 1M NaCl, 0.25 ml 1M KCl, 1 ml 2M glucose, and 1 ml 2M Mg^{2+} stock prepared from 20.33 g $MgCl_2 \cdot 6\ H_20$ and 24.65 g $MgSO_4 \cdot 7\ H_20$ in 100 ml) was added to each tube. The tubes were placed in an Innova 4300 Incubator Shaker (New Brunswick Scientific, Enfield, Connecticut) at 37ºC and 150 rpm for 1.5 hours.

The transformed cells were then inoculated onto the LB plates with ampicillin/IPTG/X-Gal. One-hundred µl of transformed culture that had been grown in SOC medium was spread onto each plate. For each sample, a total of four plates were streaked. The plates were then incubated overnight at 37ºC inverted. The next day the plates were moved to 4ºC for several hours to bring out the color of the blue colonies. The remaining cells in SOC medium were preserved by the addition of 40% v/v glycerol in water to a final concentration of 16% glycerol and then frozen and stored at -80ºC.

A.7.3 Growth of *E. coli* Colonies

Colonies from the LB plates were then transferred to media to grow the *E. coli* containing the plasmids with the DNA inserts that would be sequenced. Functional Biosciences, the company chosen to perform the DNA sequencing, recommended growing each colony in 1 ml of Terrific Broth in 96 deep-well square section blocks. Terrific Broth was prepared by dissolving 12 g tryptone, 24 g yeast extract, and 4 ml glycerol in water to a total volume of 900 ml. One-hundred ml of a 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 solution was prepared. The two solutions were autoclaved separately and then mixed. The final mixed solution was cooled, and ampicillin was added to a final concentration of 100 µg/ml.

The resulting Terrific Broth was added to 96 Square Deep Well Plates with a well volume of 2.2 ml each (Axygen, Union City, California). Eleven-hundred µl was added to each well, and the plate was covered with Breath-Easy™ sealing membrane (Diversified Biotech, Dedham, Massachusetts). The membrane served to prevent accidental inoculation of a well, and the membrane over a certain well was punctured just prior to inoculation of that well. The Terrific Broth in each well was inoculated with one white colony, which was picked from the LB agar plate with a sterile toothpick. Following inoculation of all 96 wells in the block, the

punctured membrane was removed and replaced with a new one. The membrane allowed transfer of gases while preventing contamination between wells or from the ambient air.

Four 96-well blocks of E. coli were grown at one time in the Innova 4300 Incubator Shaker. The shaker platform was modified to hold the well blocks securely, as shown in Figure A.A.3.

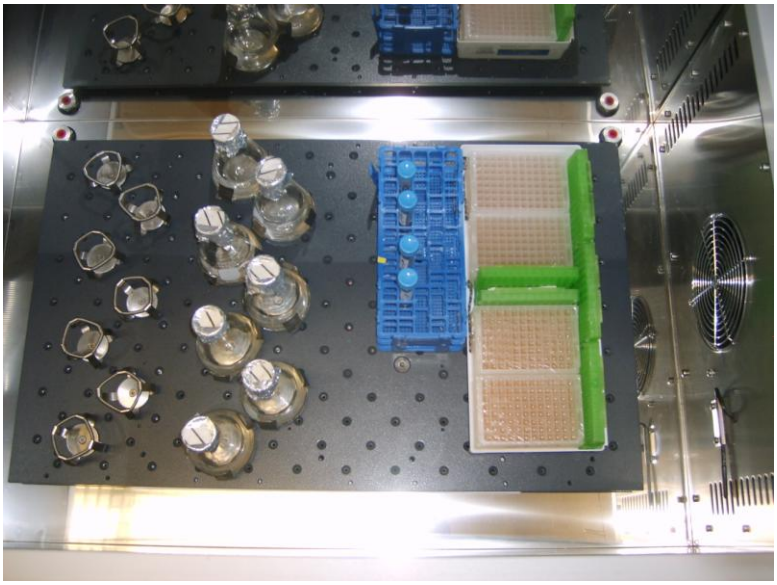

Figure A.3: Four 96 deep-well blocks inoculated with *E. coli* in the Innova 4300 Incubator Shaker.

The *E. coli* in the well blocks were grown at 37ºC and shaken at 250 rpm for 20 hours. The membrane was then carefully removed. Ninety µl of media in each well was transferred to a 96-well plate, and 60 µl of autoclaved 40% v/v glycerol in water was added to result in a final concentration of 16% glycerol. The 96-well plate was covered with foil tape, vortexed to mix the glyercol, and frozen and stored at -80ºC. The 96-well block was then covered again with membrane and centrifuged at 750x*g* for 30 minutes at 4ºC to pellet the *E. coli*. The membrane was removed and the residual media dumped off and then blotted onto a paper towel. The block was covered with foil tape and stored at -80ºC.

A.8 Preparation of Total RNA Samples for Quantitative PCR

A.8.1 DNase Treatment and Cleanup of Total RNA Samples

Before total RNA samples could be used for qPCR, several steps had to be performed. First, to make sure that quantitative data reflected only gene expression, measuring the amount of mRNA corresponding to each gene, and not any contaminating DNA, the samples were treated with DNase. Ten µg of each total RNA sample that had been previously extracted and stored at -80°C in aliquots was diluted in water to a final volume of 50 µl. Deoxyribonuclease (DNase) treatment was performed using the TURBO DNA-*free*™ kit (Ambion, Grand Island, New York) according to the manufacturer's protocol. One µl (2 U/µl) of TURBO DNase and 5 µl of 10X TURBO DNase buffer was added to the diluted RNA sample and mixed by vortexing. The sample was incubated at 37ºC for 30 minutes in a water bath. Afterward, 5 µl of DNase Inactivation Reagent was added to each sample, and the sample was vortexed. After incubation at room temperature for 2 minutes with gentle mixing, the sample was centrifuged at 10,000x*g* for 1.5 minutes to pellet the inactivation reagent. The supernatant, containing the RNA sample, was moved to a new tube, and water was added to a total volume of 100 µl per sample.

The RNA samples were further purified up and concentrated using RNA Clean & Concentrator™-5 columns (Zymo Research, Irvine, California) according to the manufacturer's protocol. Because the columns were only able to bind 5 µg of RNA each, the 100 µl of RNA per sample following DNase treatment was divided in half and processed using two columns. One-hundred µl of RNA Binding Buffer and 75 µl of absolute ethanol were added to each RNA sample that had been treated with DNase, after which the tubes were vortexed. The mixture was transferred to a spin column and centrifuged at 12,000x*g* for 1 minute; the flow- through was discarded. Four-hundred µl of RNA Prep Buffer was added to each column, after which it was centrifuged at 12,000x*g* for 1 minute and the flow-through discarded. Finally, the column was

washed twice, first with 800 µl and then with 400 µl of RNA Wash Buffer. Following addition of wash buffer in each step, the column was centrifuged at 12,000x*g* for 1 minute, and the flow-through was discarded. Then the column was centrifuged for 12,000x*g* for 2 minutes to elute any residual fluid from the washing steps. Finally, the RNA samples was eluted by added 7 µl of water directly to the column matrix, letting the column stand for 1 minute at room temperature, and centrifuging the column 10,000x*g* for 30 seconds.

A.8.2 Reverse Transcription

Following DNase treatment and cleanup, the concentration of each total RNA sample was determined using UV spectroscopy. Total RNA rather than messenger RNA (mRNA) was used because it avoided possible loss of material and degradation due to the additional step of isolating mRNA, and methods used to collect mRNA would fail to collect molecules lacking a poly-A tail (Bustin and Nolan 2009).

Reverse transcription was performed using the SMARTScribe™ Reverse Transcriptase enzyme (Clontech, Mountain View, California) according to the manufacturer's protocol. This enzyme is one of the commercially available Modified Murine Leukemia Virus reverse transcriptases, which were found to have the second-highest yield of reverse transcription among 8 different enzymes tested (Stahlberg et al. 2004). The reverse transcription yield is important because only total RNA strands that are reverse transcribed into cDNA are available for quantitative PCR, due to the ability of DNA polymerase to only amplify DNA.

Using the SMARTScribe enzyme, 3 µg of each total RNA sample was reverse transcribed in a 40 µl reaction. Four µl of oligo$(dT)_{18}$ primer (20 µM) was added to the RNA sample, and water was added to a total volume of 20 µl. The RNA and primer mixture was heated at 72ºC for 3 minutes to denature the sample and then immediately cooled on ice. Then 8 µl of 5X First-Strand Buffer, 4 µl of dNTP Mix (10 µM each dNTP) and 4 µl 20 mM

dithiothreitol (DTT) were added, and the tube contents were mixed by pipetting up and down. Finally, 4 µl of SMARTScribt RT enzyme (100 U/µl) was added, and the tube contents were mixed again. The reverse transcription reactions were incubated at 42ºC for 90 minutes in the thermal cycler. Afterward, the reaction was terminated by heating the tubes to 70ºC for 15 minutes.

Following the reverse transcription reactions, 3 µl of each sample was collected. The samples were diluted with water to a total volume of 6.75 µl, resulting in each µl containing the reverse transcription product of 40 ng of total RNA. The eight samples were mixed to be later used to prepare standards for qPCR and were stored at -20ºC. Seventy-four µl of water was added to the remaining 37 µl of each reverse transcription product, diluting the reverse transcription products so that each µl contained the reverse transcription product of 25 ng of total RNA. The diluted samples were stored at -20ºC.

A.9 Primer Design and Testing

DNA oligonucleotide primers were designed for quantitative PCR for selected genes. Gene sequences used for the primer design were a combination of sequences provided by Functional Biosystems following DNA sequencing and sequences of similar genes in other green algae. Gene and/or protein sequences from the *Chlorella* sp. were aligned with the homologous gene sequences found for green algae, with the goal of priming in conserved regions. Alignments were performed using the online software package ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Larkin et al. 2007; Goujon et al. 2010) and the stand-alone software package GeneDoc (http://www.nrbsc.org/gfx/genedoc/) (Nicholas and H.B. Nicholas 1997). These homologous sequences typically belonged to the species *Chlamydomonas reinhardtii*, *Volvox carteri*, *Ostreococcus tauri*, and *Mircomonas* sp. In the case that there was an error in the sequence for the *Chlorella* sp. obtained from the sequencing of

the subtracted library genes, use of primer binding sites in regions conserved among similar green algae would increase the likelihood that the primers would still work in qPCR.

Primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), which uses the online tool Primer 3 (Rozen and Skaletsky 2000) to design primers and then tests them for specificity against nucleic acid sequence databases using BLAST, the Basic Local Alignment Search Tool available at the National Center for Biotechnology Information on the National Institutes of Health website (Johnson et al. 2008). Settings used in Primer-BLAST were default settings, except for the following: PCR product size of 60-400, specificity check against green algae (BLAST taxid: 3041) using the nr (non-redundant) protein database, primer size of 18-25 bases with an optimal size of 20 bases, and max poly-X of 4.

Primers were synthesized by Integrated DNA Technologies (Coralville, Iowa) and were purified using standard desalting. Primer stocks of 100 µM of each primer, stored at -20ºC, and 10 µM of each primer, stored at 4ºC, were made using autoclaved, filter-sterilized TE buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA). For each gene, at least two pairs of primers were ordered. The pairs of primers were tested, and the best pair, as judged by sensitivity and selectivity to amplify only the desired product, was selected for further use. Detailed information on the primers used for qPCR is provided in Appendix C.

A.10 Quantitative PCR

The detailed materials and methods information presented herein seek to provide the information enumerated in the MIQE Guidelines, which are the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (Bustin et al. 2009).

Quantitative PCR was performed using the diluted reverse transcription products from the total RNA for each of the eight samples. For each reaction, 1 µl of template containing the reverse transcription products of 25 ng of total RNA was used. For each gene analyzed by

qPCR, a master mix of 12 µl of each primer (10 µM stocks) and 246 µl of water for a total volume of 270 µl was prepared and vortexed. Thirty-one and a half µl of this mixture was transferred to each of eight 200 µl PCR tubes, and 3.5 µl of template was added to each tube. The mixture was vortexed, and 10 µl was added to each of three tubes. Finally, 10 µl of SYBR FAST® Universal 2X qPCR Master Mix (Kapa Biosystems, Woburn, Massachusetts) was added to each tube, resulting in a final reaction volume of 20 µl. The reaction for each gene was performed in triplicate for each of the eight samples, with the exception of the reference gene actin, for which six replicates were performed for each sample. Thus each tube contained 200 nM of each primer, 1 µl of template, 8.2 µl of water, and 10 µl of qPCR master mix. A no transcript control was prepared for each gene, substituting water for the template in the reactions. Each reaction was performed in triplicate.

Reactions were performed using the MiniOpticon™ Real-Time PCR Detection System (Bio-Rad, Hercules, California) in GeneMate 8-Strip UltraFlux white PCR tubes with optically clear flat caps (Catalog Number T-3224-1; BioExpress, Kaysville, Utah). Thermal cycling conditions were 3 minutes at 95°C to activate the enzyme and 40 cycles of 3 seconds at 95°C, 20 seconds at 60°C, and 2 seconds at 72°C followed by a plate read to measure fluorescence. Immediately following thermal cycling, a melting curve was generated by heating the reactions to 95°C for 1 minute, dropping the temperature to 50°C, and raising the temperature from 50°C to 95°C in 0.2°C increments, collecting fluorescence at each step.

Data was analyzed with MJ Opticon™ Monitor software (version 3.1.32; Bio-Rad, Hercules, California). For the amplification plots, the following settings were used: Options > Subtract Baseline > Trend, Threshold > Manual > 0.007. A manual threshold of 0.007 was in the exponential phase for all of the plots. Amplification plots were smoothed with the third-least

level of smoothing. For the melting curves, the following software settings were used: Options > Subtract Baseline > Global Minimum > Temp Range > 50°C to 60°C, Peak Location Boundaries > 72°C and 95°C, and curve smoothing at the fourth-highest level. Quantification cycle (C_q) values for each sample were exported as comma-separated value files. For plate and reaction settings, the plate was specified as MJ White since white tube strips were used, and the reaction volume was set to 20 µl.

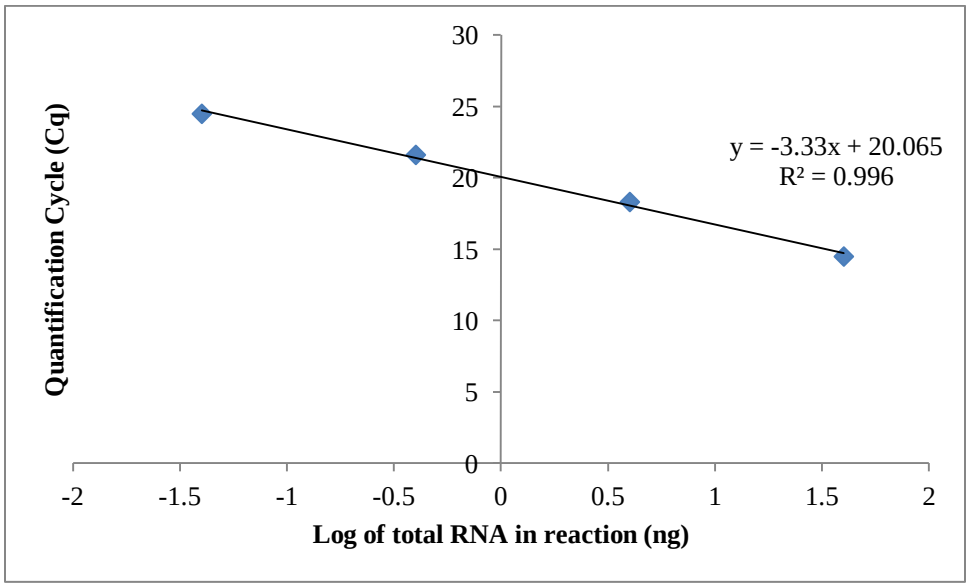

Figure A.4: Standard curve prepared from 40 ng, 4 ng, 400 pg, and 40 pg of reverse transcribed total RNA. Reaction efficiency, determined from the equation eff = $10^{-1/slope}$ -1, is 99.7%.

Standards were prepared for each gene. An equal amount of cDNA from the eight samples was mixed at a concentration of 40 ng/µl. Ten-times dilutions were made of the mixed cDNA for standards, corresponding to 40 ng, 4 ng, 400 pg, and 40 pg of reverse-transcribed total RNA per qPCR reaction. The C_q values for the reactions were plotted against the log of the RNA amount in the reaction, and a linear regression line was fitted through the points. The slope of the linear regression line was used to determine the reaction efficiency using the formula eff =

$10^{-1/slope}$-1 (Bustin et al. 2009). Quantitative PCR was only done on those reactions for which the efficiency was between 90% and 110%. An example of a standard curve is shown in Figure A.4.

APPENDIX B: DETAILED LIST OF GENES IN SUBTRACTED LIBRARIES

Table B.1 lists all of the genes matched to the expressed sequence tags from the subtracted libraries. The numbers represent the number of clones for each gene from the 96 sequenced in the library.

Table B.1: List of Genes in Subtracted Libraries

Subtracted Library	up1vs5	up5vs1	up2vs6	up6vs2	up3vs7	up7vs3	up4vs8	up8vs4
Name of Homologous Gene	**180Chl 50N**	**180CC 50N**	**180Chl 100N**	**180CC1 00N**	**400Chl 50N**	**400CC 50N**	**400Chl 100N**	**400CC1 00N**
Mitochondrial Genes	**3**	**1**	**6**	**3**	**8**	**0**	**5**	**1**
ATP synthase subunit beta	2							
ATP synthase, cf0 subunit i			2					
ATP synthase, cf1 alpha subunit				1				
ATP synthase, cf0 c subunit							1	
NADH dehydrogenase		1						
NADH dehydrogenase subunit 1			3		3		3	
NADH dehydrogenase subunit 3					1			
NADH dehydrogenase subunit 6					1			
ATP synthase d, mitochondrial				1				1
v-type proton ATP synthaase subunit d2				1				
cytochrome oxidase subunit 2					2		1	
cytochrome oxidase subunit 3	1		1		1			

(Table B.1 continued)

(Table B.1 continued)

Other Methbolism Genes	**3**	**0**	**0**	**6**	**3**	**4**	**0**	**4**
methionine-r-sulfoxide reductase	1							
glutaredoxin-like protein				1				
nucleoside diphosphate kinase					1			
glutamate decarboxylase						1		
soluble inorganic pyrophosphatase				1				
lipolytic protein g-d-s-l family					1			
isochorismatase family protein				1				
dihydrolipoamide dehydrogenase				1				
n-carbamyl-l-amino acid amidohydrolase								1
cell wall-associated hydrolase	2			1	1			
major lipid droplet protein						1		
UDP-glucose 4-epimerase/UDP-sulfoquinovose synthase						1		1
acyl-CoA binding protein								1
fumarase hydratase						1		
pyridoxine 5'-phosphate (PNP) oxidase								1
15-cis-zeta-carotene isomerase				1				
Photosystem I Genes	**11**	**7**	**19**	**6**	**14**	**2**	**23**	**7**
PS I P700 chlorophyll a apoprotein A1 (PsaA)	9		12		14		21	1
PS I subunit vi (PsaH)	1							
PS I reaction center subunit viii (PsaI)				1				
PS I reaction center subunit v (PsaG)	1		1				2	
PS I reaction center subunit xi (PsaL)		7	6	5		2		6
Photosynthetic Electron Transport Genes	**0**	**4**	**0**	**0**	**0**	**0**	**0**	**6**
ferredoxin (PetF)								2
cytochrome c6 (PetJ)		4						4
Photosystem II Genes	**15**	**3**	**11**	**2**	**18**	**1**	**17**	**6**
PSII protein D1 (PsbA)				1		1		
PS II apoprotein CP47 (PsbB)	10	1	11		10		5	
PSII protein D2 (PsbD)	1							
PS II cytochrome b559 alpha subunit (PsbE)	3				8		9	
PS II protein I (PsbI)	1							
PS II oxygen evolving protein 1 (PsbO)							3	
PSII oxygen evolving protein 3 (PsbQ)		1		1				2
PSII 10 kDa protein(PsbR)								2
PS II protein W (PsbW)		1						2

(Table B.1 continued)

(Table B.1 continued)

Other Chloroplast Genes	**1**	**3**	**1**	**0**	**0**	**0**	**4**	**5**
light-harvesting chlorophyll a/b-binding	1	3					4	5
photochlorophyllide reductase subunit n			1					
Transcription/Translation Factor Genes	**12**	**3**	**11**	**1**	**8**	**1**	**5**	**1**
eukaryotic translation elongation factor 1 alpha 2		1						
eukaryotic translation initiation factor 3 subunit 3				1				
translational initiation factor 1							1	
protein translation factor sui1								1
Cytochrome p450-like TATA box binding protein	12	2	7		8	1	4	
rRNA promoter binding protein			4					
30S Ribosomal Genes	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**
30S ribosomal protein 1 (chloroplast)					1			
40S Ribosomal Genes	**1**	**2**	**2**	**4**	**1**	**4**	**0**	**4**
40S ribosomal protein S3				2				
40S ribosomal protein S9	1							
40S ribosomal protein S12								1
40S ribosomal protein S13		1	1	2		1		1
40S ribosomal protein S16		1			1	1		
40S ribosomal protein S23						1		
40S ribosomal protein S24			1					1
40S ribosomal protein S29						1		1
60S Ribosomal Genes	**6**	**6**	**1**	**2**	**4**	**8**	**3**	**19**
60s acidic ribosomal protein P2								2
60S ribosomal protein L9		1				2		
60S ribosomal protein L10a-1	1						1	
60S ribosomal protein L11	2							
60S ribosomal protein L12	1							
60S ribosomal protein L13				1				
60S ribosomal protein L16			1					
60S ribosomal protein L23							1	
60S ribosomal protein L23a		1		1		4		9
60S ribosomal protein L29		2						
60S ribosomal protein L30					1	2		2
60S ribosomal protein L31	1						1	
60S ribosomal protein L33								2
60S ribosomal protein L34								1
60S ribosomal protein L37a	1	2			1			1

(Table B.1 continued)

(Table B.1 continued)

60S ribosomal protein L38					1			
60S ribosomal protein L39								2
60S ribosomal protein L44					1			
Other Protein Synthesis Genes	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**0**
RNA polymerase							1	
spliceosomal protein fbp11 splicing factor prp40							1	
Protein Destination and Storage Genes	**1**	**1**	**0**	**8**	**1**	**1**	**1**	**0**
ubiquinol-cytochrome c reductase complex protein					1			
heat shock protein 90				2				
heat shock protein 81-2				1				
chaperonin cpn10				1				
ubiquitin fusion protein	1							
ubiquitin extension protein		1						
ubiquitin-fold modifier 1				1				
atp-dependent clp protease adaptor protein				3				
rna recognition motif-containing protein						1		
signal peptidase complex subunit 3b							1	
Intracellular Trafficking Genes	**1**	**0**	**0**	**3**	**1**	**2**	**0**	**0**
translocator protein	1							
adp-ribosylation factor						1		
ran-family small GTPase				1	1			
GTP-binding nuclear protein ran-3-like						1		
phosphate/phosphoenolpyruvate translocator				1				
charged multivesicular body protein 5				1				
Protein Phosphatase Genes	**0**	**0**	**0**	**1**	**0**	**1**	**0**	**0**
phosphoprotein phosphatase				1				
protein phosphatase 2a subunit a3						1		
Other Signal Transduction Genes	**1**	**0**	**0**	**2**	**0**	**0**	**0**	**0**
sulfate adenylyltransferase				1				
guanylyl cyclase				1				
auxin-binding protein 1	1							
Disease Genes	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**
senescence-associated protein		1						
Transposons	**1**	**0**	**0**	**0**	**3**	**0**	**1**	**0**
retrotransposon protein	1				3		1	
Other Genes	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**
low co2 inducible gene [*C. reinhardtii*]				1				
Unknown Genes	**33**	**56**	**39**	**51**	**23**	**59**	**25**	**36**

APPENDIX C: ADDITIONAL INFORMATION ON PRIMERS USED IN QUANTITATIVE PCR

Table C.1: Detailed Information on Primers Used for qPCR Reactions

Gene	Primers (forward then reverse; each listed 5' to 3')	T_m (°C)	Efficiency	R^2 of Standard Curve	Amplicon Length (bp)	Amplicon T_m (°C)
Photosystem II P680 apoprotein CP47	AGGTGTAGCAGCAGCCCACATTG TGTCGCGTCCCATGACGGAG	61.9 62.0	96.06%	0.9738	283	80.4
60S ribosomal protein L23a	AAGCCCCGCTATTCGGTGGT GGCACGGATGTCGACGATGAACA	61.7 61.5	98.03%	0.9999	198	83.0
cytochrome b559 alpha subunit	TGTCTGGTGCTACAGGAGAACGC ACGGTCTGTAATAAGCGGGGTTTCT	61.0 60.0	108.6%	0.9993	206	79.0
Photosystem I Reaction Center XI	GGACGCACTCTCTCCCCGGAT GCCCAGGCAACTCCGGAAAGT	63.3 62.5	104.4%	0.9651	98	84.4
ATP synthase subunit alpha	AAACAGTCTTCGTCGCCCACGC GAGACACGGCCCAGACGCTT	62.4 62.4	105.3%	0.9930	199	82.6
molecular chaperone (HSP family)	AGCTGTGTCCCGAGTGGATGGG GTGGAGCGGTAGCGCAGCAAA	63.4 62.9	97.24%	0.9809	268	83.6
light-harvesting chlorophyll-a/b binding	CAGGTCACCCGCGCCTCTGT CACCGAGCATAGCCCAGCGTG	64.8 63.0	99.66%	0.9960	202	86.0
phosphoprotein phosphatase	TGCATTCACGGTGGTCTTTCCCCTG CCAGCCAGCACCTCTTGGACTGA	63.2 63.2	106.5%	0.9995	162	82.0
oil globule associated protein	ATTGATGGAAGCTGCACGCCCC AGACGCTACCACGCTGTCGTGA	62.8 62.6	98.60%	0.9988	79	80.0

Detailed information for the primers used for quantitative PCR are presented above in Table C.1. The primer melting temperatures given were supplied by the primer manufacturer, Integrated DNA Technologies (Coralville, Iowa). Reaction efficiencies and R^2 values are from

standard curves. The amplicon melting temperatures are from the melting curves generated after the qPCR reactions.

VITA

John Joseph Tate was born and raised in Baton Rouge, Louisiana, attending St. Aloysius School and Catholic High School. In college, he studied a wide variety of subjects and obtained a Bachelor of Science in mathematics at Louisiana State University in May 2006. Combining an appreciation for chemistry with an aptitude for numbers, he commenced his studies in chemical engineering, completing his Master of Science in the field in May 2012 at Louisiana State University.